Iron-sulfur (Fe-S) clusters are essential cofactors, and mitochondria contain several Fe-S proteins, including the [4Fe-4S] protein aconitase and the [2Fe-2S] protein ferredoxin. Fe-S cluster assembly of these proteins occurs within mitochondria. Although considerable data exist for yeast mitochondria, this biosynthetic process has never been directly demonstrated in mammalian mitochondria. Using [35S]cysteine as the source of sulfur, here we show that mitochondria isolated from Cath.A-derived cells, a murine neuronal cell line, can synthesize and insert new Fe–35S clusters into aconitase and ferredoxins. The process requires GTP, NADH, ATP, and iron, and hydrolysis of both GTP and ATP is necessary. Importantly, we have identified the 35S-labeled persulfide on the NFS1 cysteine desulfurase as a genuine intermediate en route to Fe-S cluster synthesis. In physiological settings, the persulfide sulfur is released from NFS1 and transferred to a scaffold protein, where it combines with iron to form an Fe-S cluster intermediate. We found that the release of persulfide sulfur from NFS1 requires iron, showing that the use of iron and sulfur for the synthesis of Fe-S cluster intermediates is a highly coordinated process. The release of persulfide sulfur also requires GTP and NADH, probably mediated by a GTPase and a reductase, respectively. ATP, a cofactor for a multifunctional Hsp70 chaperone, is not required at this step. The experimental system described here may help to define the biochemical basis of diseases that are associated with impaired Fe-S cluster biogenesis in mitochondria, such as Friedreich ataxia.
Transferred to apoproteins, forming holo and active proteins (2–4).

Studies of Fe-S cluster biogenesis in mammalian mitochondria are relatively recent and have focused primarily on the effects of in vivo depletion of a particular component of the mitochondrial Fe-S cluster machinery on Fe-S protein activities (12–14). However, this method has many limitations because of variable degrees of protein depletion and secondary effects occurring during the depletion time course (9). Furthermore, in intact mammalian cells, it is not possible to manipulate critical molecules (e.g. nucleotides) to determine their roles in Fe-S cluster assembly without compromising other essential metabolic processes. Therefore, it has been difficult to dissect different steps of the mitochondrial Fe-S cluster biogenesis in vivo. Ideally, such studies must be performed with mitochondria isolated from mammalian cells. However, so far, there has been no experimental system available to study Fe-S cluster assembly in isolated mammalian mitochondria.

Here we present the results of studies using isolated and metabolically active mammalian mitochondria supplemented with [35S]cysteine as the source of sulfur for Fe-S cluster synthesis. The experimental system resembles, in some respects, the system we developed for studying Fe-S cluster synthesis in yeast mitochondria (15–17). We found that murine CAD4 mitochondria were able to generate an activated form of sulfur derived from cysteine and bound to the mitochondrial NFS1 (15). The supernatant fractions contained the precursor protein depleted mitochondria, the NFS1-bound persulfide sulfur was not released, even in the presence of added ATP and iron. Likewise, in iron-depleted mitochondria, the NFS1-bound persulfide sulfur was not released, even in the presence of added GTP, NADH, and ATP. In the presence of GTP, NADH, and iron but no ATP, the persulfide sulfur was released, but radiolabeled aconitase was not detected. Only in the presence of adequate levels of all four constituents (GTP, NADH, iron, and ATP) was the precursor product relationship observed. That is, the radiolabeled NFS1 persulfide was decreased greatly, and radiolabeled aconitase was formed. The use of persulfide sulfur and iron for Fe-S cluster assembly was closely coordinated by processes requiring GTP, NADH, and ATP.

**Experimental Procedures**

**Bacterial Expression of Proteins**—The plasmids pZM2 and pZM4 were provided by Dr. Silke Leimkühler (18). The first 55 amino acids of the NFS1 precursor protein were deleted, generating NFS1Δ1–55, and the plasmid pZM2 codes for this truncated version of NFS1 with an N-terminal His tag and an ampicillin-selectable marker. The plasmid pZM4 codes for human ISD11 (with no His tag) and a chloramphenicol-selectable marker. The plasmids pZM2 and pZM4 were cotransformed into BL21 (DE3) cells (Stratagene). Cells carrying both plasmids were grown in Luria-Bertani (LB) medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol to A600 of ~0.6. Coexpression of His₆-NFS1 and ISD11 was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. The NFS1-ISD11 complex was purified by nickel-nitrilotriacetic acid-agarose chromatography as described for purification of the corresponding yeast complex (19, 20).

The codon-optimized and synthetic genes for human FDX1 and FDX2 precursor proteins in the pMAT vector were obtained from Invitrogen. The plasmid pMAT/pFDX1 was digested with Nco1 and Xho1, and the fragment containing the FDX1 ORF was cloned into the same sites of pET21d (Novagen). Likewise, the Nde1-FDX2 ORF-Xho1 fragment of pMAT/pFDX2 was cloned into pET21b. The resulting constructs, pET21d/pFDX1 and pET21b/pFDX2, incorporate a C-terminal His tag into the respective precursor proteins. BL21 (DE3) codon plus cells carrying the plasmid pET21d/pFDX1 or pET21b/pFDX2 were grown in LB medium containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) to A600 of ~0.6. Protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Both proteins were solubilized with 8 M urea in 50 mM Tris/HC1 (pH 8.0) and centrifuged at 250,000 × g for 20 min at 20 °C to remove insoluble materials (15). The supernatant fractions contained the precursor protein (FDX1 or FDX2) and were used for import and Fe-S cluster loading assays.

**Cell Cultures and Isolation of Mitochondria**—The mouse Cath.A-derived (CAD) catecholaminergic cell line was provided by Dr. Eldo V. Kuzhikandathil (21). The CAD cells were cultured in DMEM/F12 medium supplemented with 8% FBS and 100 units/ml penicillin/streptomycin in a humidified incubator at 5% CO₂. Cells were grown to about 80–90% confluency and processed for mitochondria isolation. The human cervical carcinoma cell line (HeLa) was grown in DMEM supplemented with 2% glutamine, 10% FBS, and 100 units/ml penicillin/streptomycin.

Mitochondria were isolated from CAD or HeLa cells essentially as described previously (22) with some modifications. Briefly, cells were washed with Ca²⁺/Mg²⁺-free PBS (pH 7.4), transferred to a 50-ml Falcon tube, and centrifuged at 600 × g for 10 min. The cell pellet thus obtained was resuspended in ice-cold mitochondria isolation buffer (MIB) (20 mM Hepes/KOH (pH 7.5), 0.25 M sucrose, 1 mM EDTA, 1 mM DTI, 0.1 mg/ml BSA, 10 units/ml Trasylol, and 0.5 mM PMSF). The cell suspension was gently passed through a 27-gauge needle, followed by Dounce homogenization (25 strokes). The homogenate was diluted with MIB and centrifuged at 2,000 × g for 5 min. The supernatant was transferred to a different tube and saved. The pellet was resuspended in MIB and subjected to a repeat of Dounce homogenization, followed by centrifugation at 2,000 × g for 5 min. The supernatant obtained was combined with the previously saved supernatant and centrifuged at 10,000 × g for 12 min. The resulting mitochondrial pellet was resuspended in a small volume of HS buffer (20 mM Hepes/KOH (pH 7.5) and 0.6 M sorbitol) containing 0.5 mM PMSF, stored on ice, and used for various assays within 2 h.
Fe-S Cluster Assembly in Mammalian Mitochondria

Fe-S Cluster Assembly—Insertion of newly formed Fe-S clusters into an endogenous apoprotein by mammalian mitochondria was examined as follows. When necessary, mitochondria were preincubated at 30 °C for 10 min to deplete endogenous nucleotides and NADH (16). A typical assay mixture (100 μl) contained mitochondria (200 μg of proteins) in buffer A (20 mM Hepes/KOH (pH 7.5), 0.6 mM sorbitol, 10 mM Mg(OAc)₂, and 40 mM KOAc) containing 0.1 mg/ml BSA and 1 mM DTT. As needed, reaction mixtures were supplemented with various combinations of ATP, GTP, an energy-regenerating system containing 20 mM phosphocreatine and 0.2 mg/ml creatine kinase (EGS), NADH, α-ketoglutarate (αKG), and/or ferrous ascorbate (Fe²⁺). Where indicated, addition of the “master mix” produced final concentrations of ATP (4 mM), GTP (1 mM), EGS (20 mM phosphocreatine and 0.2 mg/ml creatine kinase), and NADH (2 mM) in the assay. Following addition of [³⁵S]cysteine (10 μCi, 1.075 Ci/mmole), samples were incubated at 30 °C for different time periods (5–60 min). Reaction mixtures were diluted 10-fold with ice-cold HS buffer, and mitochondria were recovered by centrifugation at 15,000 × g for 10 min at 4 °C. The pellet was resuspended in 35 μl of 50 mM Tris/HCl (pH 8.0) containing 0.5 mM PMSF. To rupture mitochondrial membranes, samples were frozen at −80 °C, followed by thawing and bath sonication for 15 s at 4 °C. This process was repeated three times. Samples were centrifuged at 15,000 × g for 30 min at 4 °C, and the supernatant fractions containing soluble proteins of mitochondria were analyzed by native PAGE followed by autoradiography.

An assay involving simultaneous import and Fe-S cluster assembly of ferredoxins was performed as follows. Samples contained mitochondria (200 μg of proteins) in buffer A containing BSA (0.1 mg/ml), αKG (1 mM), ATP (4 mM), GTP (1 mM), NADH (2 mM), DTT (1 mM), and [³⁵S]cysteine (10 μCi). The reaction was initiated by adding urea-denatured apoFDX1 or apoFDX2 precursor protein (100 ng). The total volume was 100 μl, and the final urea concentration was 160 mM. Samples were incubated at 30 °C for 45 min and then diluted with ice-cold HS buffer. Mitochondria were recovered by centrifugation at 15,000 × g for 10 min at 4 °C and analyzed by native PAGE followed by autoradiography as described above. Some assays were also performed in two steps, separating ferredoxin import and Fe-S cluster assembly, as indicated in the legend for Fig. 7.

Persulfide Formation by the Cysteine Desulfurase—Nucleotide/NADH-depleted mitochondria (100–200 μg of proteins) were mixed with 10 μCi [³⁵S]cysteine in buffer A containing 0.1 mg/ml BSA in a final volume of 50 μl (20). After incubation at 30 °C for 20–30 min, reaction mixtures were diluted 10-fold with ice-cold HS buffer containing 0.15 M NaCl. Mitochondria were recovered by centrifugation at 15,000 × g for 10 min at 4 °C. Samples were analyzed by non-reducing SDS-PAGE followed by autoradiography. The persulfide formation by the purified human NFS1-ISD11 complex was evaluated essentially as described for the yeast complex (20). Briefly, the purified NFS1-ISD11 complex was added to HS buffer containing 0.15 mM pyridoxal phosphate, 0.15 M NaCl, and 10 μCi [³⁵S]cysteine in a final volume of 50 μl. After incubation at 30 °C for 15 min, proteins were precipitated with ice-cold 10% trichloroacetic acid. Samples were centrifuged at 15,000 × g for 30 min at 4 °C, and the protein pellets were analyzed by non-reducing SDS-PAGE followed by autoradiography.

For immunoprecipitation experiments, nucleotide/NADH-depleted mitochondria were incubated with [³⁵S]cysteine at 30 °C for 20 min and recovered by centrifugation as described above. The pellet was solubilized with the immunoprecipitation (IP) buffer (50 mM Tris/HCl (pH 7.5), 1% Triton X-100, 0.15 M NaCl, and 1 mM PMSF), and the mitochondrial lysate thus obtained was centrifuged at 15,000 × g for 2 min at 4 °C to remove insoluble material, if any. The lysate was added to protein A-Sepharose with already bound anti-human NFS1 antibodies (Santa Cruz Biotechnology). Samples were incubated at 4 °C for 3 h with end-over-end mixing, and the beads were washed with IP buffer. The bound proteins were eluted with SDS loading buffer containing no DTT and were analyzed by non-reducing SDS-PAGE followed by autoradiography. The purified NFS1-ISD11 complex incubated with [³⁵S]cysteine (see above) served as a positive control for IP.

Miscellaneous—Aconitase activity was evaluated by an in-gel assay or a spectrophotometric assay as described previously (15, 23). Antibodies against human aconitase were raised in rabbits using bacterially expressed and purified ACO2. As necessary, radiolabeled protein bands in autoradiographs were quantitated using the National Institutes of Health software ImageJ.

RESULTS

Fe-S Cluster Assembly by Isolated and Intact Mammalian Mitochondria—Mitochondria were isolated from CAD cells and supplemented with [³⁵S]cysteine as the source of sulfur and ferrous ascorbate as a source of iron. Reaction mixtures were incubated with a master mix including nucleotides (ATP and GTP), NADH, and an EGS consisting of phosphocreatine and creatine kinase. Mitochondria were recovered, and mitochondrial membranes were ruptured. Following centrifugation, the supernatant fractions containing the soluble proteins were analyzed by native PAGE followed by autoradiography. An endogenous soluble protein was found to be strongly radiolabeled, and the radiolabeling occurred in a time-dependent manner (Fig. 1A, lanes 3–6). In the absence of master mix, very little, if any, radiolabeling of the protein was observed regardless of the incubation time (Fig. 1A, lanes 1 and 2). Therefore, addition of the master mix was essential for radiolabeling of the protein. As discussed later, the radiolabeled protein was identified as aconitase (ACO2).

In a separate experiment, mitochondria were first treated with chloramphenicol, an inhibitor of organellar protein synthesis. Samples were supplemented with [³⁵S]cysteine, iron, and master mix, and then incubated. Radiolabeling of ACO2 was not affected by the presence of chloramphenicol (Fig. 1A, compare lanes 7 and 8 with lanes 3 and 6), indicating that the ACO2 signal was not due to covalent incorporation of [³⁵S]cysteine into the polypeptide backbone. This notion is consistent with the observation that the [³⁵S] label associated with ACO2 was completely lost when samples were analyzed by SDS-PAGE (instead of native PAGE) after incubation with [³⁵S]cysteine (data not shown, but see Fig. 9).

A. Dancis and D. Pain, unpublished data.
The radiolabeling of ACO2 was not due to exchange of $^{35}$S into existing clusters of ACO2. For example, when Fe-$^{35}$S clusters were formed and inserted into ACO2, incubation of mitochondria with a large excess of unlabeled cysteine did not remove the radiolabel from ACO2 (Fig. 1B). Finally, the master mix and time-dependent Fe-S cluster synthesis was not restricted to CAD mitochondria and was also observed with mitochondria isolated from HeLa cells (Fig. 1C). These results suggest that mitochondria isolated from mammalian cell lines are metabolically active. They contain a complete machinery for Fe-S cluster biogenesis and are capable of forming new Fe-S clusters and inserting them into a population of endogenous apoACO2 in a manner that is dependent on added nucleotides (ATP and GTP) and NADH.

The Radiolabeled Endogenous Protein in Mammalian Mitochondria Is Aconitase—We suspected the radiolabeled protein in CAD mitochondria to be aconitase (ACO2) because, in isolated yeast mitochondria, aconitase (Aco1) can be labeled with Fe-$^{35}$S clusters under similar conditions (Fig. 1A, lanes 9 and 10) (15). However, the radiolabeled protein in murine CAD mitochondria migrated significantly slower than the radiolabeled yeast Aco1 (Fig. 1A). By contrast, the radiolabeled protein in human HeLa mitochondria migrated close to and slightly faster than yeast Aco1 (Fig. 1C). To address these issues, CAD, HeLa, and yeast mitochondrial lysates were analyzed by two identical native gels side by side. After electrophoresis, one gel was processed directly for an in-gel aconitase activity assay (Fig. 2A, left panel). The other gel was processed for immunoblotting.
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A. Aconitase activity (Native gel)

B. Immunoblot (SDS gel)

C. Aconitase activity

FIGURE 2. Identification of the radiolabeled protein in mammalian mitochondria as aconitase (ACO2). A, experiments were performed with mitochondria (Mito) isolated from CAD, HeLa, and yeast cells. Mitochondrial lysates were analyzed by two native gels. One gel was processed directly for in-gel aconitase activity (left panel). The other gel was processed for immunoblotting and probed with anti-human ACO2 antibodies (right panel). B, mitochondrial proteins were separated by SDS-PAGE and analyzed by immunoblotting using anti-human ACO2 antibodies. C, CAD mitochondria (1% or less) increase in aconitase activity was observed, implying that the pool of apoACO2 was further characterized by comparison of aconitase activity/protein in CAD mitochondria migrated much slower than yeast aconitase, whereas the HeLa aconitase migrated close to yeast aconitase. However, no such difference in aconitase migration (yeast versus CAD or HeLa samples) was detected when mitochondrial proteins were analyzed by SDS-PAGE followed by immunoblotting using anti-ACO2 antibodies (Fig. 2B). The calculated molecular masses of the mature forms of CAD ACO2 (83.3 kDa), HeLa ACO2 (83.3 kDa), and yeast Aco1 (83.7 kDa) are almost identical, consistent with their indistinguishable mobilities in SDS gels (Fig. 2B). However, the calculated isoelectric points of the mature proteins are significantly different: 7.4 (CAD), 6.75 (HeLa), and 7.06 (yeast). Compared with the higher pl of CAD ACO2, the lower pl of HeLa ACO2 and yeast Aco1 may explain their more rapid migration toward the positively charged electrode in native gels (Figs. 1A and 2A). Together, these results suggest that the radiolabeled protein in mammalian mitochondria (CAD or HeLa) is aconitase (ACO2). This notion was further substantiated as follows.

Isolated CAD mitochondria were incubated with $[^{35}S]$cysteine in the presence of added iron and master mix. Mitochondria were recovered, and soluble proteins were analyzed by native gel. Following electrophoresis, the gel was first processed for aconitase activity (Fig. 2C, left panel). After drying, the same gel was exposed to film for autoradiography (Fig. 2C, right panel). The band exhibiting aconitase activity was superimposable with the radiolabeled band. Furthermore, increasing levels of both activity and radiolabeling were observed with increasing amounts of mitochondria. We conclude that the radiolabeled protein in mammalian mitochondria (CAD or HeLa) is aconitase. An intact [4Fe-4S] cluster is essential for aconitase activity, and, therefore, the radiolabeled aconitase most likely represents the holo form of the enzyme with newly inserted clusters (ACO2 [4Fe-4$^{35}$S]).

The $[^{35}$S] labeling of aconitase in our assays does not represent the repair of damaged [3Fe-4S] clusters to functional [4Fe-4S] clusters because this would require incorporation of (unlabeled) iron and not (radiolabeled) sulfur. How then did the protein (ACO2) become radiolabeled? Most likely, mammalian mitochondria as isolated contain a pool of apoACO2 serving as a substrate for newly made $[^{35}$S]-labeled clusters. Aconitase is one of the most abundant proteins in mitochondria, and the existence of apoACO2 in isolated mitochondria may reflect incomplete cluster loading of aconitase in vivo. When isolated mitochondria were incubated with $[^{35}$S]cysteine and master mix, radioactive sulfur was generated and utilized for Fe-$^{35}$S cluster synthesis within the organelle. Newly formed Fe-$^{35}$S clusters were subsequently incorporated into endogenous apoACO2. Analysis of the samples by native PAGE and autoradiography allowed direct visualization of the labeled protein.

The pool of apoACO2 was further characterized by comparing aconitase activity in CAD mitochondria before and after Fe-S cluster loading. Specifically, intact CAD mitochondria were incubated with unlabeled cysteine (20 μM) and iron (10 μM) in the absence or presence of master mix, as was done for the radiolabeling experiments. Mitochondria were recovered, and the aconitase activity was measured by a quantitative spectrophotometric assay (15). On the basis of the data presented in Fig. 1A, unlabeled Fe-S clusters were likely inserted into endogenous apoACO2 in samples containing the master mix and not in samples lacking the master mix. However, only a marginal (1% or less) increase in aconitase activity was observed, implying that the pool of endogenous apoACO2 in isolated CAD mitochondria is quite small. Nevertheless, the small amount of apoACO2 was sufficient for our radiolabeled Fe-S cluster loading assays.

Individual Requirements of ATP and GTP for [4Fe-4S] Cluster Assembly—Isolated CAD mitochondria were incubated at 30°C for 10 min to deplete endogenous nucleotides and NADH. These mitochondria were then incubated with $[^{35}$S]cysteine in the absence or presence of added ATP, GTP, and/or EGS. All reaction mixtures were supplemented with NADH and iron. Very little radiolabeling of ACO2 was observed in the presence of ATP alone (Fig. 3A, lane 2) or GTP alone (Fig. 3A, lane 3), and it was greatly enhanced when ATP, GTP and EGS were included together (Fig. 3A, lane 4). These results suggest that efficient Fe-S cluster biogenesis of ACO2 in
isolated mammalian mitochondria requires both ATP and GTP. The implication is that added ATP and GTP are transported into the mitochondrial matrix and are utilized there for Fe-S cluster assembly. The mode of ADP/ATP transport via the ADP/ATP carrier across the inner membrane of mitochondria is conserved from yeast to mammals (24, 25). By contrast, GTP/GDP carrier Ggc1 mediates the exchange of cytosolic GTP for matrix GDP across the mitochondrial inner membrane (26). An analogous protein to the yeast Ggc1 may therefore exist in mammalian mitochondria, allowing transport of added GTP into the matrix. Alternatively, GTP might be transported into the matrix nonspecifically through other carrier proteins in the inner membrane of mammalian mitochondria. These issues remain to be resolved.

We then performed experiments under conditions that allow metabolic generation of nucleotides (ATP and GTP) in the mitochondrial matrix. Briefly, nucleotide/NADH-depleted mitochondria were supplemented with αKG, NADH, and iron and incubated with [35S]cysteine. Strikingly, significant radiolabeling of ACO2 was observed with as low as 0.5 mM αKG even though no ATP or GTP was added (Fig. 3A, lane 5). In fact, the signal obtained with 1 mM αKG alone (Fig. 3A, lane 6) was very similar to the combined effects of 4 mM ATP, EGS, and 2 mM GTP (Fig. 3A, lane 4). Furthermore, addition of both ATP (4 mM) and GTP (2 mM) only slightly increased the stimulatory effects of 1 mM αKG alone (Fig. 3A, compare lanes 6 and 7). As indicated above, these experiments were performed with mitochondria that were preincubated at 30 °C for 10 min to deplete endogenous nucleotides and NADH (Fig. 3A, lanes 1–7). Note that such a treatment did not alter the amount of available endogenous apoACO2, as judged by the levels of ACO2 [Fe-35S] formed (Fig. 3A, compare lanes 7 and 8). The results with αKG can be explained as follows.

When added to intact mitochondria, αKG can efficiently enter into the matrix by the 2-oxoglutarate transporter and/or the dicarboxylate carrier protein in the inner membrane (27) and be utilized by αKG dehydrogenase to form succinyl-CoA (28). Succinyl-CoA, in turn, is converted to succinate by succinyl-CoA synthetase (SCS): A-SCS, which generates ATP, and G-SCS, which generates GTP (Fig. 3B). The two isoforms share a common α subunit but have distinct β subunits (29). The β

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**FIGURE 3. Hydrolysis of both ATP and GTP is required for Fe-S cluster assembly in mitochondria.** A, isolated CAD mitochondria were preincubated at 30 °C for 10 min to deplete endogenous nucleotides and NADH (lanes 1–7). As a control, one aliquot of mitochondria was left on ice without any preincubation at 30 °C (lane 8). Mitochondria were then supplemented with ATP (4 mM), GTP (2 mM), EGS, and/or αKG (0.5–1 mM) in buffer A containing 0.1 mg/ml BSA and 1 mM DTT as indicated. After addition of NADH (2 mM), [35S]cysteine (10 μCi) and ferrous ascorbate (10 μM), reaction mixtures were incubated at 30 °C for 45 min. Samples were analyzed by native PAGE followed by autoradiography. B, schematic showing that isolated and intact mammalian mitochondria, when supplemented with αKG, are capable of generating ATP and GTP via the Krebs cycle. Suc-CoA, succinyl-CoA. C, CAD mitochondria were preincubated with ATPγS (1–2 mM) or GTPγS (1–2 mM) for 10 min on ice. Following addition of αKG (1 mM), NADH (2 mM), [35S]cysteine (10 μCi), and ferrous ascorbate (10 μM), samples were incubated at 30 °C for 45 min and analyzed as in A. D, CAD mitochondria were preincubated with ATPγS or GTPγS for 10 min on ice, and then GTP or ATP was added as indicated. Following addition of αKG (1 mM), NADH (2 mM), [35S]cysteine (10 μCi), and ferrous ascorbate (10 μM), samples were incubated and analyzed as in C.
subunit confers the adenine or guanine specificity. Under our experimental conditions, both ATP and GTP were generated from added αKG, thereby promoting Fe-S cluster biogenesis of ACO2 even in the absence of directly added ATP or GTP. These results substantiate our conclusion that both ATP and GTP in the matrix are required for Fe-S cluster biogenesis in mammalian mitochondria.

To further delineate the individual roles of ATP and GTP, assays were performed in the presence of poorly hydrolyzable nucleotide analogs. The radiolabeling of endogenous apoACO2 in the presence of added αKG was strongly inhibited by ATPγS or GTPγS (Fig. 3C). Furthermore, direct addition of GTP failed to block inhibition by ATPγS (Fig. 3D, lanes 1–3). Likewise, directly added ATP did not rescue inhibition by GTPγS (Fig. 3D, lanes 4–6). We conclude that hydrolysis of both ATP and GTP is necessary for Fe-S cluster biogenesis of aconitase in mammalian mitochondria. These nucleotides are individually required, perhaps as cofactors for distinct Fe-S cluster assembly components and/or during different stages of Fe-S cluster biogenesis. Therefore, addition of GTP or ATP cannot circumvent processes that require ATP hydrolysis or GTP hydrolysis, respectively.

_NADH Requirement for [4Fe-4S] Cluster Assembly—_The Fe-S cluster insertion experiments described above were performed with added NADH. To determine whether NADH is necessary, assays were performed with or without NADH addition. In mitochondria as isolated, efficient radiolabeling of aconitase was observed even in the absence of added NADH, and there was no stimulatory effect by added NADH (Fig. 4A). However, the situation was very different when mitochondria were first incubated at 30 °C for 10 min to deplete endogenous nucleotides and NADH and then supplemented with ATP, GTP, EGS, iron, and [35S]cysteine. In the absence of added NADH, the radiolabeling of endogenous ACO2 was poor, and it was greatly enhanced by the addition of NADH in a concentration-dependent manner (Fig. 4B, lanes 2–4). Mitochondria as isolated appeared to contain sufficient levels of endogenous NADH to support Fe-S cluster assembly in our assays. Upon incubation at 30 °C for 10 min, endogenous NADH was likely reduced below the threshold level, thereby requiring added NADH for efficient Fe-S cluster biogenesis. Therefore, neither nucleotides (ATP and GTP) nor NADH alone are sufficient for [4Fe-4S] cluster biogenesis of aconitase. All three components (ATP, GTP, and NADH) are individually required for efficient Fe-S cluster assembly in mitochondria.

The source of nicotinamide adenine dinucleotide (oxidized or reduced) in mitochondria is not fully understood (30). These dinucleotides may be transported from the cytoplasm to mitochondria and/or synthesized within the organelle. For example, NAD transporters have been identified in the inner membrane of yeast and plant mitochondria (31, 32). Some evidence for NAD transport into mammalian mitochondria also exists (33, 34), although a specific transporter has yet to be identified. On the basis of the stimulatory effects of added NADH in our assays, we speculate that mitochondria were able to transport NADH in some form across the inner membrane to the site of Fe-S cluster synthesis (i.e., the matrix). The transport could be mediated by dedicated but unknown transporter(s) or nonspecifically by other carrier proteins in the inner membrane of mitochondria. A potential alternative to mitochondrial transport of cytoplasmic NAD/NADH is the synthesis of these molecules within the organelle. Interestingly, an isoform of NMN adenylyltransferase (NMAT3) has been found in the matrix of mammalian mitochondria, and some studies suggest that mitochondria utilize this enzyme to synthesize NAD from NMN that is transported from the cytoplasm (30, 35). However, as in the case for NAD/NADH, no NMN-specific transporter has been identified. In any case, addition of NMN (together with ATP, GTP, and EGS) failed to promote Fe-S cluster biogenesis in CAD mitochondria (Fig. 4B, lane 6). Likewise, addition of NADPH was also ineffective (Fig. 4B, lane 5), consistent with its poor transport into mitochondria (17, 36). In summary, the stimulatory effects on [4Fe-4S] cluster biogenesis of aconitase were observed only when NADH was added, and neither NMN nor NADPH could substitute for NADH.

[35S]Labeling of Aconitase in Mitochondria Is Dependent on Iron Availability—Fe-S cluster assembly should require iron. All of the Fe-S cluster insertion experiments described above were performed with added ferrous ascorbate, although the physiological iron ligand that supplies iron for Fe-S cluster biogenesis in mitochondria is unknown. Surprisingly, efficient radiolabeling of ACO2 was observed even without any added

![Image](https://example.com/image.png)
iron, and added iron had a small stimulatory effect (Fig. 5A). Most likely, mitochondria as isolated contained a sufficient pool of stored iron and were able to efficiently use this endogenous iron for new Fe-S cluster synthesis. To substantiate this notion, intact mitochondria were treated with a membrane-permeable chelator, o-phenanthroline, washed, and then incubated with [35S]cysteine, αKG, ATP, GTP, and NADH with or without added ferrous ascorbate. No radiolabeling of ACO2 was detected in the absence of added iron (Fig. 5B, lane 3). More importantly, the process was recovered to a greater extent when iron was added (Fig. 5B, lanes 4 and 5). These results suggest that the chelator o-phenanthroline, by virtue of its ability to cross the inner membrane of mitochondria, bound and immobilized iron from a stored pool in the matrix. Consequently, after treatment with o-phenanthroline, endogenous iron was no longer available for Fe-S cluster synthesis. The inhibitory effect was reversed by iron addition. Added iron was imported into the matrix, new Fe-35S clusters were made, and ACO2 became radiolabeled. Similar experiments with yeast mitochondria demonstrated the existence of an iron storage pool in the mitochondrial matrix, which could be detected by Fe-S cluster assembly assays (19) or, more directly, by Mossbauer measurements of non-heme, high-spin, ferrous iron pool (37). In summary, isolated mammalian mitochondrial matrix can utilize endogenous or imported iron for Fe-S cluster biosynthesis.

[2Fe-2S] Cluster Assembly of Imported Ferredoxins—Mitochondria contain [2Fe-2S] proteins, such as ferredoxins, in addition to [4Fe-4S] proteins, such as aconitase. In fact, there are two different ferredoxins in mammalian mitochondria, FDX1 and FDX2 (38, 39). Like ACO2, both ferredoxins are localized to the mitochondrial matrix. However, unlike in the case for ACO2, 35S labeling of endogenous ferredoxins was not observed. The lack of ferredoxin labeling could be due to technical issues such as lower levels of FDX1 and FDX2 apoproteins in mitochondria. Alternatively, different pathways might be involved for the biogenesis of [2Fe-2S] and [4Fe-4S] cluster proteins in mitochondria. To test these possibilities, the precursor forms of FDX1 and FDX2, each with a C-terminal His6 tag, were expressed in bacteria. The proteins were found to be sequestered in inclusion bodies. After solubilization with 8 M urea, the proteins were found to be highly pure (Fig. 6A). We then asked whether CAD mitochondria were able to use imported apoFDX1 and/or apoFDX2 as substrates for [2Fe-2S] cluster biogenesis. Briefly, mitochondria were incubated with unlabeled FDX1 or FDX2 precursor protein in the presence of [35S]cysteine, αKG, ATP, GTP, and NADH. Fe-35S clusters were found to be efficiently inserted into endogenous ACO2 as well as imported FDX1 or FDX2 (Fig. 6B, lanes 2 and 3). As a control, mitochondria were pretreated with valinomycin to dissipate the membrane potential across the inner membrane, thereby blocking the import capability (17). These mitochondria were then incubated with FDX1 or FDX2 precursor protein in the presence of [35S]cysteine, αKG, ATP, GTP, and NADH. Only ACO2 was radiolabeled and not FDX1 or FDX2 (Fig. 6B, lanes 4 and 5). Under these conditions, FDX1/FDX2 import did not occur, and, hence, no radiolabeling of FDX1/FDX2 was observed. We conclude that, as in intact cells, biogenesis of both [2Fe-2S] and [4Fe-4S] clusters can occur simultaneously in our in vitro assay system with isolated mitochondria.

Individual Requirements of ATP, GTP, and NADH for [2Fe-2S] Cluster Assembly—The experiments described above were performed in one step and involved simultaneous import and Fe-S cluster assembly of ferredoxins (Fig. 6B). Protein import into the matrix requires nucleotides (40). To determine the nucleotide requirements specifically for the cluster biogenesis of imported ferredoxins, experiments were performed in two steps, separating import from Fe-S cluster insertion. In the first step, unlabeled apoFDX1 precursor protein was imported into isolated CAD mitochondria in the presence of ATP with no added GTP or NADH. Mitochondria were recovered by centrifugation, removing unimported precursor molecules and excess ATP. In the second step, mitochondria with imported FDX1 were supplemented with αKG plus GTP or ATP. Samples were then incubated with [35S]cysteine and NADH. The radiolabeling of FDX1 that occurred in the presence of αKG and GTP was strongly inhibited by ATPγS (Fig. 7A, compare lanes 2 and 3). Likewise, the observed FDX1 radiolabeling in the presence of αKG and ATP was greatly diminished by GTPγS (Fig. 7A, compare lanes 4 and 5). Similar results were also obtained with imported FDX2 (data not shown). Furthermore,
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FIGURE 6. Fe-35S labeling of newly imported ferredoxins. A. the precursor forms of human ferredoxins (FDX1 and FDX2), each with a C-terminal His\textsubscript{6} tag, were expressed in bacteria. The proteins were solubilized from inclusion bodies with 8 M urea and analyzed by SDS-PAGE followed by Coomassie Blue staining of the gel. Std, prestained protein standards with their molecular mass in kilodalton. B. CAD mitochondria were incubated with or without valinomycin (5 \mu M) for 5 min on ice. Mitochondria were then supplemented with \alpha-KG (1 mM), ATP (4 mM), GTP (1 mM), NADH (2 mM), and [35S]cysteine (10 \mu Ci). The FDX1 (100 ng) or FDX2 (100 ng) precursor protein was added as indicated. After incubation at 30 °C for 45 min, samples were analyzed by native PAGE followed by autoradiography.

the inhibitory effects of nucleotide analogs on radiolabeling of imported FDX1/FDX2 and endogenous ACO2 were very similar. These results suggest that hydrolysis of both ATP and GTP is required for [2Fe-2S] as well as [4Fe-4S] cluster assembly.

To determine the NADH requirement, apoFDX1 or apoFDX2 precursor protein was first imported into mitochondria in the presence of ATP as described above. During import at 30 °C for 15 min, endogenous NADH was most likely depleted (e.g. see Fig. 4B). Mitochondria were recovered and then incubated with \alpha-KG, ATP, GTP, and [35S]cysteine in the absence or presence of added NADH. As in the case for endogenous ACO2, radiolabeling of imported FDX1 or FDX2 was found to be greatly enhanced by the addition of NADH (Fig. 7B, compare lanes 1 and 2 and lanes 3 and 4). Therefore, both the [2Fe-2S] and [4Fe-4S] cluster assembly processes are stimulated by NADH addition. In summary, these results provide direct evidence that the Fe-S cluster assembly machinery in isolated and intact mammalian mitochondria can handle proteins with different clusters in a similar nucleotide- and NADH-dependent manner.

Identification of the NFS1-bound Persulfide in Mitochondria—Cysteine desulfurases are essential for the viability of eukaryotic cells because they perform a vital role in Fe-S cluster synthesis (41). Specifically, these enzymes abstract sulfur from the substrate cysteine, generating a covalent persulfide on the active site cysteine of the enzyme (Enz-S-SH) (19, 20, 42). In physiological settings, the persulfide sulfur is utilized for Fe-S cluster synthesis. NFS1 is the only known cysteine desulfurase in mammalian mitochondria, and we sought to demonstrate NFS1-bound persulfide formation in mitochondria.

NFS1 interacts and forms a stable complex with a small protein, ISD11, in mitochondria (12, 43, 44). To obtain the active enzyme complex, a N-terminally truncated form of NFS1 (first 55 amino acids deleted) with a N-terminal His\textsubscript{6} tag (His\textsubscript{6}-NFS1) and ISD11 (18) were expressed together in bacteria and purified by nickel-nitritotriacetic acid-agarose affinity chromatography (19, 20). ISD11 without a His tag was copurified with His\textsubscript{6}-NFS1, and the NFS1-ISD11 complex thus obtained was more than 90% pure (data not shown). To assess persulfide formation, the purified enzyme complex was incubated with [35S]cysteine and analyzed by non-reducing SDS-PAGE followed by autoradiography. NFS1 was found to be radiolabeled, indicating covalent persulfide formation on the enzyme (NFS1-S-S\textsubscript{35SH}) (Fig. 8A, top panel, lane 1). These results allowed us to use the purified NFS1-ISD11 complex as a positive control for studies of the NFS1 activity in mitochondria as described below.

The persulfide-forming activity of the purified enzyme complex did not require any nucleotides or NADH (e.g. Fig. 8A, top panel, lane 1). However, new Fe-S cluster synthesis/assembly in nucleotide/NADH-depleted mitochondria was strictly dependent on nucleotide and NADH addition (e.g. Figs. 3A, 4B, and 7, A and B). Nucleotides/NADH may therefore be required for downstream events such as synthesis of Fe-S cluster intermediates on the scaffold protein and/or transfer of these intermediates from the scaffold to apoproteins in mitochondria. In that case, the NFS1-bound persulfide may be detected under conditions that inhibit transfer of the persulfide sulfur to downstream recipients, therefore “trapping” the persulfide sulfur on the enzyme in mitochondria. To test this possibility, nucleotide/NADH-depleted mitochondria were incubated with [35S]cysteine in the absence of any added nucleotides or NADH, and the total mitochondrial proteins were analyzed by non-reducing SDS-PAGE followed by autoradiography. A major radiolabeled protein (~46 kDa) was detected, and the radiolabeling was most likely due to the covalent [35S]persulfide formation on NFS1 in mitochondria (Fig. 8A, top panel, lane 2). Chloramphenicol did not interfere with the persulfide formation on NFS1 (Fig. 8A, top panel, compare lanes 2 and 3). These results are consistent with the observation that blocking mitochondrial protein synthesis with the addition of chloramphenicol did not affect the overall Fe-S cluster biogenesis of aconitase (Fig. 1A). Furthermore, as in the case for the purified NFS1-ISD11 complex (data not shown), treatment with DTT prior to SDS-PAGE released the persulfide sulfur, and radiolabeled NFS1 was no longer detected in mitochondria (Fig. 8A, top panel, lane 4). The radiolabeled band in mitochondria rep-
represents the authentic mature NFS1 with bound persulfide. It migrated close to but slightly faster than the [35S]persulfide-labeled His6-NFS1 generated by the purified NFS1/H18528 ISD11 complex (Fig. 8A, top panel, lane 1). The exact signal sequence cleavage site of NFS1 in mitochondria has not yet been determined, and, therefore, the authentic mature form of NFS1 in mitochondria could be slightly smaller than the bacterially expressed NFS1/H9004 1–55. Also, the authentic mitochondrial NFS1 lacks a His6 tag. The identities of His6-NFS1 of the purified complex and NFS1 in mitochondria were confirmed by immunoblotting using anti-NFS1 antibodies (Fig. 8A, bottom panel, compare lanes 1 and 2). Finally, the identity of the radiolabeled 46-kDa protein in mitochondria as NFS1 was further confirmed by IP of the protein (Fig. 8B). Therefore, the radiolabeled 46-kDa protein in mammalian mitochondria is indeed the NFS1 cysteine desulfurase, and the 35S radiolabel associated with NFS1 represents [35S]persulfide formation on the enzyme.

Fe-S cluster biogenesis of ACO2 was strongly inhibited by the presence of ATPS or GTPS (Fig. 3, C and D). We therefore sought to determine the effects of these non-hydrolyzable nucleotide analogs on NFS1-bound persulfide. Briefly, nucleotide/NADH-depleted mitochondria were incubated with ATP (4 mM) and incubated with FDX1 (100 ng) or FDX2 (100 ng) precursor protein at 30 °C for 15 min (first step). Mitochondria were recovered and resuspended as in A and then supplemented with αKG (1 mM), GTP (1 mM), ATP (4 mM), and [35S]cysteine (10 μCi). As indicated, NADH (2 mM) was added. After incubation at 30 °C for 45 min (second step), samples were analyzed by native PAGE and autoradiography.

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FIGURE 7. ATP hydrolysis, GTP hydrolysis, and NADH are individually required for [2Fe-2S] cluster assembly of ferredoxins. A, CAD mitochondria were supplemented with ATP (4 mM) and then incubated with the FDX1 precursor protein (100 ng) at 30 °C for 15 min (first step). Reaction mixtures were diluted with HS buffer and centrifuged, and mitochondrial pellets were resuspended in buffer A containing 0.1 mg/ml BSA and 1 mM DTT. As indicated, samples were supplemented with αKG (1 mM), GTP (1 mM), ATP (4 mM), NADH (2 mM), ATPγS (2 mM), and/or GTPγS (2 mM). After addition of [35S]cysteine (10 μCi), reaction mixtures were incubated at 30 °C for 45 min (second step). Mitochondria were recovered, and samples were analyzed by native PAGE followed by autoradiography. B, CAD mitochondria were supplemented with ATP (4 mM) and incubated with FDX1 (100 ng) or FDX2 (100 ng) precursor protein at 30 °C for 15 min (first step). Mitochondria were recovered, and samples were analyzed by native PAGE and autoradiography.

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The effects of these analogs on the NFS1-bound persulfide in mitochondria can be explained as follows. After preincubation at 30 °C, mitochondrial nucleotides were depleted to some extent. The depletion was not absolute, and the residual nucleotides likely permitted utilization of a portion of the NFS1-bound persulfide sulfur for a basal level of Fe-S cluster synthesis. In the presence of increasing concentrations of nucleotide analogs, the residual nucleotides became increasingly unavailable for persulfide sulfur utilization. Consequently, the persulfide remained completely “frozen” on NFS1, leading to increased radiolabeling of the enzyme.

**FIGURE 8. Identification of the cysteine desulfurase-bound persulfide in mammalian mitochondria.**

A. Top panel, nucleotide/NADH-depleted CAD mitochondria (Mito, 100 µg of proteins, lanes 2–4) were incubated with or without chloramphenicol (0.1 mg/ml) for 10 min on ice and then mixed with 10 µCi [35S]cysteine in buffer A containing 0.1 mg/ml BSA. Following incubation at 30 °C for 20 min, samples were diluted with HS buffer containing 0.15 M NaCl and centrifuged to recover mitochondria. The mitochondrial pellets were solubilized with SDS loading buffer with or without DTT (10 mM) as indicated. The bacterially expressed and purified NFS1-ISD11 complex (50 ng) was incubated with 10 µCi [35S]cysteine in HS buffer containing 0.15 mM pyridoxal phosphate and 0.15 M NaCl for 15 min at 30 °C. After trichloroacetic acid precipitation, the protein pellet was solubilized with SDS loading buffer containing no reducing agents. Samples were analyzed by SDS-PAGE followed by autoradiography. Bottom panel, the NFS1-ISD11 complex (5 ng) and identical mitochondrial samples as above were analyzed by immunoblotting using anti-human NFS1 antibodies. B. Nucleotide/NADH-depleted CAD mitochondria and the purified NFS1-ISD11 complex were incubated separately with [35S]cysteine as in A. Four sets of reactions were performed. One set was analyzed with no further treatment (Total). The other sets were subjected to IP using anti-human NFS1 antibodies (αNFS1), no antibodies (no antibody), or antibodies against Mir1, an unrelated yeast mitochondrial protein (αMir1) (19). Samples were analyzed by a non-reducing SDS gel followed by autoradiography. C. As indicated, nucleotide/NADH-depleted CAD mitochondria were preincubated with ATPγS and/or GTPγS for 10 min on ice. Samples were then incubated with [35S]cysteine (10 µCi) at 30 °C for 20 min, and mitochondria were recovered and analyzed by non-reducing SDS-PAGE followed by autoradiography.
Utilization of the NFS1-bound Persulfide Sulfur for Fe-S Cluster Synthesis—Nucleotide/NADH-depleted CAD mitochondria were incubated with [35S]cysteine in the absence or presence of the master mix (ATP/GTP/EGS/NADH) and iron. One set of samples was analyzed for NFS1-S-35SH formation by non-reducing SDS-PAGE and autoradiography (Fig. 9A, top panel). Another set of identical samples was evaluated for Fe-35S cluster loading of ACO2 by native PAGE and autoradiography (Fig. 9A, bottom panel). The 35S labeling of NFS1 (Fig. 9A, top panel, lane 1) was reduced by more than 90% with the addition of master mix (Fig. 9A, top panel, lane 2), and very little radiolabeled NFS1 was detected when iron was included as well (Fig. 9A, top panel, lane 3). More importantly, radiolabeling of aconitase was increased in the corresponding samples (Fig. 9A, bottom panel, lanes 1–3). Therefore, the signal on NFS1 reflecting persulfide formation and the signal on aconitase reflecting new Fe-S cluster synthesis varied reciprocally, indicating a precursor-product relationship (i.e., transfer of the persulfide sulfur from NFS1 to downstream Fe-S cluster recipients such as aconitase).

When mitochondria were pretreated with o-phenanthroline to deplete endogenous iron stores, the [35S]persulfide on NFS1 persisted even after addition of the master mix (Fig. 9A, top panel, compare lanes 4 and 5) and no radiolabeling of aconitase was detected (Fig. 9A, bottom panel, lanes 4 and 5). The NFS1-bound persulfide sulfur was not utilized because of lack of iron and remained trapped. In fact, when iron was included together with the master mix, radiolabeled NFS1 was no longer detected (Fig. 9A, top panel, lane 6) and aconitase became radiolabeled (Fig. 9A, bottom panel, lane 6). Addition of iron together with the master mix allowed new Fe-35S clusters to be synthesized, and the persulfide sulfur was used in the process. We conclude that the NFS1-bound persulfide sulfur as described here represents a bona fide biochemical intermediate en route to Fe-S cluster synthesis in mitochondria.

To extend these findings, we performed a similar experiment with αKG, allowing mitochondria to metabolically generate ATP and GTP inside the organelle. Briefly, nucleotide/NADH-depleted mitochondria (endogenous iron present) were incubated with [35S]cysteine in the absence or presence of αKG and NADH, and/or iron. In the presence of αKG alone, there was no reduction in radiolabeled NFS1 (aconitase, Fig. 9B, top panel, compare lanes 1 and 2), and, accordingly, there was very little radiolabeling of aconitase (Fe-S cluster assembly, Fig. 9B, bottom panel, lane 2). When NADH was included together with αKG, the NFS1-bound persulfide was decreased by more than 90% regardless of added iron (Fig. 9B, top panel, lanes 3 and 4), and aconitase became radiolabeled (Fig. 9B, bottom panel, lanes 3 and 4). Note that endogenous iron was present in mitochondria of these samples (Fig. 9B, top and bottom panels, lanes 1–4). In a separate experiment, nucleotide/NADH-depleted mitochondria were pretreated with o-phenanthroline to minimize the availability of endogenous iron. When these mitochondria were incubated with [35S]cysteine, the radiolabeled persulfide was generated on NFS1. However, the persulfide completely persisted even in the presence of αKG and NADH, and no radiolabeled aconitase was detected (Fig. 9B, top and bottom panels, lanes 5–7). Only when iron was included together with αKG and NADH was the persulfide level greatly reduced with concomitant appearance of ACO2 [Fe-35S] (Fig.
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![Graph showing Fe-S cluster assembly](Image)

**FIGURE 10.** GTP, NADH, and iron together are required for the release of NFS1-bound persulfide sulfur. A, nucleotide/NADH-depleted CAD mitochondria (100 μg of proteins) were incubated with [35S]cysteine (10 μCi) at 30 °C for 20 min (first step). Samples were diluted with HS buffer and centrifuged, and the mitochondrial pellets thus obtained were resuspended in buffer A containing 0.1 mg/ml BSA. As indicated, ATP (4 mM), GTP (1 mM), NADH (2 mM), ATPγS (2 mM), and ferrous ascorbate (10 μM) were added. Samples were incubated at 30 °C for 20 min (second step), and mitochondria were recovered and analyzed by autoradiography. B, nucleotide/NADH-depleted CAD mitochondria (100 μg of proteins) were incubated with 2 mM o-phenanthroline (o-Phe) for 10 min on ice. Samples were diluted with HS buffer and centrifuged, and the mitochondrial pellets thus obtained were resuspended in buffer A containing 0.1 mg/ml BSA and incubated with [35S]cysteine (10 μCi) at 30 °C for 20 min (first step). Reaction mixtures were diluted with HS buffer and centrifuged. The mitochondrial pellets were resuspended in buffer A containing 0.1 mg/ml BSA and, as indicated, supplemented with ATP (4 mM), GTP (1 mM), NADH (2 mM), and ferrous ascorbate (10 μM). After incubation at 30 °C for 20 min (second step), samples were analyzed by non-reducing SDS-PAGE followed by autoradiography.

9B, top and bottom panels, lane 8). Therefore, utilization of the NFS1-bound persulfide sulfur for Fe-S cluster assembly of aconitase in mammalian mitochondria requires all of the four constituents: ATP, GTP, NADH, and iron.

**Release of the NFS1-bound Persulfide Sulfur Requires GTP, NADH, and Iron but Not ATP**—For the experiments described above (e.g. Fig. 9), [35S]cysteine was present throughout the assay, allowing multiple rounds of Fe-S cluster synthesis under optimum conditions (i.e. in the presence of adequate levels of ATP, GTP, NADH, and iron). In these assays, persulfide was formed continuously on NFS1, and NFS1-bound persulfide sulfur was also continuously released and transferred to the scaffold protein for Fe-S cluster synthesis. The persulfide formation on NFS1 did not require any nucleotides, NADH, or iron. To determine how the release of NFS1-bound persulfide sulfur might be regulated, experiments were performed under conditions that allowed only a single round of persulfide formation and release. Specifically, the assays were performed in two steps. Nucleotide/NADH-depleted mitochondria (endogenous iron present) were incubated with [35S]cysteine to generate [35S]persulfide on NFS1 (first step). Mitochondria were recovered, removing free [35S]cysteine. These mitochondria with NFS1-bound [35S]persulfide were then supplemented with ATP, GTP, NADH, and/or ATPγS in various combinations and incubated with added iron (second step). The data show that the [35S]persulfide signal was completely unaffected in the presence of ATP alone (Fig. 10A, lane 2), GTP alone (Fig. 10A, lane 3), or NADH alone (Fig. 10A, lane 4), indicating no release of the radiolabeled persulfide sulfur. Likewise, ATP plus GTP (Fig. 10A, lane 5) or ATP plus NADH (Fig. 10A, lane 6) also completely failed to promote release of the persulfide sulfur. Strikingly, the signal associated with NFS1 [35S]persulfide was lost only in the presence of GTP and NADH, indicating release of the persulfide sulfur under these conditions (Fig. 10A, lane 7). This release process was not inhibited by the presence of ATPγS (Fig. 10A, lane 9). Therefore, the release of NFS1-bound persulfide sulfur specifically requires GTP and NADH and not ATP. Under these conditions (with iron present), the persulfide sulfur was most likely transferred to the ISCU scaffold protein to combine with iron for Fe-S cluster synthesis. The ISCU-bound Fe-[35S]cluster intermediates thus generated were probably destroyed by SDS, and, hence, no radiolabeled band corresponding to ISCU was detected after SDS-PAGE in our assays. A similar experiment was then performed with nucleotide/NADH-depleted mitochondria after pretreatment with o-phenanthroline to deplete endogenous iron within mitochondria. These mitochondria were incubated with [35S]cysteine to generate the radiolabeled persulfide on NFS1 (first step). After removal of [35S]cysteine, samples were incubated with ATP, GTP, and/or NADH in various combinations in the absence or presence of added iron (second step). When no iron was added, the persulfide persisted even in the presence of ATP, GTP, and NADH (Fig. 10B, lane 5). Only when iron was included with GTP and NADH was the [35S]persulfide sulfur released completely, leading to loss of the radioactive signal on NFS1 (Fig. 10B, lane 4). As long as GTP, NADH, and iron were present, addition of ATP was completely unnecessary for the persulfide release (Fig. 10B, compare lanes 4 and 6). We conclude that persulfide sulfur is not released from NFS1 (and, hence, that sulfur cannot be incorporated into the intermediate) without iron availability, therefore indicating a high degree of coordination between the utilization of these two Fe-S components. At this stage, the regulatory mechanism likely involves a GTPase and an NADH-requiring reductase. An ATPase may then par-
ticipate in transferring Fe-S cluster intermediates from the scaffold to apoproteins such as apoACO2, apoFDX1, and apoFDX2.

DISCUSSION

Fe-S cluster cofactors are essential for the function of numerous proteins. In eukaryotic cells, several Fe-S proteins, such as aconitase [4Fe-4S] and ferredoxin [2Fe-2S], are found in mitochondria, and the synthesis and assembly of these Fe-S clusters occur within the organelle. Numerous studies have been performed to investigate Fe-S cluster biogenesis in yeast mitochondria over the past two decades. By contrast, the biosynthetic process in mammalian mitochondria is receiving more attention recently, and many aspects of the process remain poorly understood (9). In fact, until now, no method has been available to directly study Fe-S cluster biogenesis in mitochondria isolated from any mammalian cells. Here, for the first time, we report new Fe-S cluster assembly by isolated and metabolically active mammalian mitochondria. An important feature of our assays is that they could be performed with intact mitochondria, thereby permitting manipulations of the organelle milieu. This novel system allowed us to identify some of the key regulatory aspects of Fe-S cluster biogenesis in mammalian mitochondria.

We found that mitochondria isolated from murine CAD cells contain a complete machinery that can efficiently synthesize new Fe-S clusters and insert them into apoproteins. These mitochondria can handle Fe-S cluster assembly of protein substrates with different types of clusters. Specifically, assembly of [4Fe-4S] clusters on endogenous aconitase and [2Fe-2S] clusters on newly imported ferredoxins can occur simultaneously and in a similar manner (Figs. 6B and 7). Therefore, the process of Fe-S cluster biogenesis in isolated mammalian mitochondria faithfully recapitulates many aspects of the in vivo process taking place in cells. Importantly, our data show a regulatory relationship between nucleotide availability and Fe-S cluster biogenesis. For example, the overall process of Fe-S cluster assembly is greatly enhanced by the addition of ATP and GTP. Isolated mammalian mitochondria do not appear to contain adequate levels of endogenous ATP/GTP to support efficient Fe-S cluster biogenesis, thereby requiring addition of these nucleotides (Fig. 1A). The requirements for directly added ATP and/or GTP, however, can be bypassed when mitochondria are supplemented with αKG (Fig. 3A), thereby promoting metabolic generation of these nucleotides in the matrix, most likely by the ATP-specific (A-SCS) and GTP-specific (G-SCS) isoforms of SCS (Fig. 3B) (45). Hydrolysis of both ATP and GTP is essential for [2Fe-2S] as well as [4Fe-4S] cluster biogenesis, and addition of ATP cannot circumvent the processes that require GTP hydrolysis and vice versa (Figs. 3, C and D, and 7A). These nucleotides are needed separately, possibly during different stages of Fe-S cluster biogenesis (see below).

Some of the critical steps in Fe-S cluster biogenesis are thought to involve chemical reduction i.e. donation of electrons (see below). NADH and/or NADPH supplied to reductase enzymes in the matrix are the best candidates for providing the necessary reducing equivalents (7, 17). However, a role for NAD(P)H in Fe-S cluster assembly by mammalian mitochon-

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Fe-S cluster biogenesis in mammalian mitochondria can be divided into three stages. Stage 1 involves persulfide formation on the cysteine desulfurase NFS1. This process does not require any nucleotides, NADH, or iron (19, 20). Cysteine binds to NFS1, and frataxin plays a role by exposing cysteine binding sites (46). NFS1 interacts with NFS1 and promotes persulfide formation (46). In stage 2, iron and sulfur are combined, generating Fe-S cluster intermediates on the scaffold protein ISCU (49). A GTpase and a reductase appear to be involved at this stage, requiring GTP and NADH, respectively. Frataxin may also play a role in this stage by providing iron or coordinating iron and sulfur donation to ISCU (47, 58). The GTpase involved has not yet been identified. The reductase is likely to be adrenodoxin reductase, which reduces ferredoxins FDX1 and FDX2 (38, 39). The reducing equivalents for this electron transport chain may derive from NADPH (50), and, therefore, an NAD(H) kinase may also be required inside mitochondria (17, 51).

The substrate that receives electrons is unknown but could be the S0 or iron involved in Fe-S cluster intermediate formation (4). In stage 3, Fe-S cluster intermediates are transferred from the scaffold protein to apoproteins such as aconitase or ferredoxins. Chaperones are involved at this stage, requiring ATP hydrolysis (55). The cochaperone Hsc20 binds hol ISCU and initiates binding to the mitochondrial Hsp70, HspA9 (54). The Hsp70 reaction cycle requires ATP hydrolysis and mediates release and transfer of Fe-S cluster intermediates to glutaredoxins and apoproteins (49). If mitochondrial levels of GTP, NADH, or iron are inadequate, then the NFS1-bound persulfide remains trapped regardless of ATP levels. Only in the presence of sufficient levels of all four constituents (i.e., GTP, NADH, iron, and ATP) is the persulfide sulfur released from NFS1 and utilized for [2Fe-2S] and [4Fe-4S] cluster assembly. See text for details.

The presence of GTP, NADH, and iron individually, and the process did not require ATP (Fig. 10). However, only in the presence of optimal levels of all four constituents together (GTP, NADH, iron, and ATP) was the NFS1-bound persulfide sulfur chased from the enzyme and subsequently utilized for Fe-S cluster assembly of aconitase (Fig. 9).

Fe-S cluster biogenesis in mammalian mitochondria is a multistep process requiring many proteins. For brevity, the process can be divided into three key stages: stage 1, persulfide formation on NFS1; stage 2, formation of Fe-S cluster intermediates on the ISCU scaffold; and stage 3, transfer of cluster intermediates to apoproteins (Fig. 11). We propose that these distinct stages have different nucleotide requirements. In stage 1, the NFS1 cysteine desulfurase abstracts sulfur from the substrate cysteine, generating a persulfide on a conserved cysteine residue of the active site of the enzyme (NFS1-S-SH). This stage does not appear to require nucleotides, NADH, or iron (Fig. 9) (20, 46). However, the cysteine desulfurase activity is highly regulated by protein-protein interactions, and it seems that at least two separate conformational changes must occur in the enzyme for optimum activity. One change is mediated by frataxin interaction, exposing the substrate-binding sites and enhancing the binding of cysteine. A second change is mediated by an ISD11 interaction that brings the bound substrate cysteine and the active site cysteine in proximity for persulfide formation (46). In stage 2, the persulfide sulfur is released and transferred from NFS1 to a scaffold protein, ISCU, and assembled with iron to form an Fe-S cluster intermediate (3, 4). As described above, the release of persulfide sulfur occurs only in the presence of adequate levels of iron, NADH, and GTP (Fig. 10B). The source of iron has not been determined, but frataxin may play a role in iron donation for forming the ISCU intermediates (47). Importantly, this stage likely involves some critical reduction steps. For example, the sulfane sulfur (S0) of the NFS1-bound persulfide must be converted to sulfide (S2-) for incorporation into the nascent cluster. Likewise, iron must also be maintained in reduced form because only ferrous iron is useful for the assembly process. Furthermore, as in the case for bacterial IscU (48), the reductive coupling of two distinct [2Fe-2S] clusters to a single [4Fe-4S] cluster may also occur on mammalian ISCU, requiring additional electrons. One or more of these steps may be mediated by reductases such as ferredoxin reductase, and NADPH may be needed as electron supplier for the reductase. The enzyme provides electrons to ferredoxin(s) and then, probably, to other components of an Fe-S cluster assembly complex, thereby allowing cluster synthesis on ISCU.

In human mitochondria, there are two different ferredoxins (FDX1 and FDX2), which are very similar. An earlier study suggested that only FDX2, and not FDX1, is required for Fe-S cluster assembly (38). However, a more recent study showed that both FDX1 and FDX2 are involved (39). In any case, the stimulatory effect of added NADH on Fe-S cluster biogenesis in our assays (Fig. 4B) is most likely due to its use for the ferredoxin reductase/ferredoxin redox chain. On one hand, the mammalian ferredoxin reductase prefers NADPH to NADH as electron supplier (50). On the other hand, the inner membrane of mitochondria is impermeable to cytosolic NADPH (36), consistent with the observation that directly added NADPH did not show any stimulation of cluster biogenesis in our assays with intact mitochondria (Fig. 4B). Therefore, NADPH must be made locally in the mitochondrial matrix. The observed stimulatory effects of NADH on cluster biogenesis of aconitase could be due to NADH, NADPH, or both. In the matrix, NADH may be converted to NADPH by a matrix-localized NAD(H) kinase. The NAD(H) kinases are the sole enzymes able to convert NAD(H) to NADP(H) using ATP as a phosphate donor. Such a kinase has been identified recently in mammalian mitochondria (51, 52), although a possible functional connection between the kinase and Fe-S cluster biogenesis remains to be determined. By contrast, the corresponding NADH kinase in the yeast mitochondrial matrix, called Po5, has been characterized, and it seems that NADPH, generated by Po5, plays an important role in Fe-S cluster biogenesis in yeast mitochondria (17, 53).

In addition to NADP(H), GTP may also be required for efficient assembly of Fe-S cluster intermediates on ISCU. The NFS1-bound persulfide signal was enhanced in the presence of GTPyS or ATPyS individually (Fig. 3C), suggesting that the processes that require GTP hydrolysis or ATP hydrolysis for

![Figure 11. Model for nucleotide- and NADH-dependent Fe-S cluster assembly in mammalian mitochondria. Fe-S cluster biogenesis in mitochondria is a multistep process requiring many proteins. For brevity, the process can be divided into three key stages: stage 1, persulfide formation on the cysteine desulfurase NFS1. This process does not require any nucleotides, NADH, or iron (19, 20). Cysteine binds to NFS1, and frataxin plays a role by exposing cysteine binding sites (46). NFS1 interacts with NFS1 and promotes persulfide formation (46). In stage 2, iron and sulfur are combined, generating Fe-S cluster intermediates on the scaffold protein ISCU (49). A GTpase and a reductase appear to be involved at this stage, requiring GTP and NADH, respectively. Frataxin may also play a role in this stage by providing iron or coordinating iron and sulfur donation to ISCU (47, 58). The GTpase involved has not yet been identified. The reductase is likely to be adrenodoxin reductase, which reduces ferredoxins FDX1 and FDX2 (38, 39). The reducing equivalents for this electron transport chain may derive from NADPH (50), and, therefore, an NAD(H) kinase may also be required inside mitochondria (17, 51). The substrate that receives electrons is unknown but could be the S0 or iron involved in Fe-S cluster intermediate formation (4). In stage 3, Fe-S cluster intermediates are transferred from the scaffold protein to apoproteins such as aconitase or ferredoxins. Chaperones are involved at this stage, requiring ATP hydrolysis (55). The cochaperone Hsc20 binds hol ISCU and initiates binding to the mitochondrial Hsp70, HspA9 (54). The Hsp70 reaction cycle requires ATP hydrolysis and mediates release and transfer of Fe-S cluster intermediates to glutaredoxins and apoproteins (49). If mitochondrial levels of GTP, NADH, or iron are inadequate, then the NFS1-bound persulfide remains trapped regardless of ATP levels. Only in the presence of sufficient levels of all four constituents (i.e., GTP, NADH, iron, and ATP) is the persulfide sulfur released from NFS1 and utilized for [2Fe-2S] and [4Fe-4S] cluster assembly. See text for details.](image-url)
Fe-S cluster biogenesis lie downstream of stage 1. Interestingly, the maximum NFS1-bound persulfide signal was obtained in the presence of both GTP-γS and ATP-γS (Fig. 8C). Most likely, GTP hydrolysis and ATP hydrolysis occur at different stages and not during the same stage of Fe-S cluster assembly. This notion is supported by the observation that GTP, and not ATP, was specifically needed for the release of NFS1-bound persulfide sulfur (Fig. 10). We postulate that GTP plays an important role during synthesis of Fe-S cluster intermediates on the scaffold protein (15). At this stage, we can only speculate how a GTPase might be involved during stage 2. For example, the GTP hydrolysis mediated by a GTPase in the mitochondrial matrix might be required for trafficking of the persulfide sulfur from NFS1 to ISCU. Alternatively, the GTP hydrolysis might facilitate the insertion of ferrous ions into ISCU for the assembly of Fe-S cluster intermediates. In any case, the GTPase remains to be identified, and more work is needed to determine the precise role of GTP in Fe-S cluster assembly in mitochondria.

In Stage 3, Hsp70 chaperones that are known to utilize ATP are involved. The cochaperone HSC20 (Jac1 in yeast) binds ISCU (Isu1 in yeast) and forms a complex with the generic mitochondrial Hsp70 chaperone, HSPA9 (Ssq1 in yeast) (54). The cochaperone and the scaffold cooperatively stimulate the ATPase activity of the Hsp70 chaperone, which may be essential for the release of scaffold-bound cluster intermediates with concomitant transfer to the recipient apoprotein (55). Such a scenario may then explain the absolute requirement for ATP hydrolysis in our assays for Fe-S cluster biogenesis of aconitase and ferredoxins in isolated mammalian mitochondria (Figs. 3, C and D, and 7A). In addition to the transfer process (stage 3), other ATP-requiring steps in Fe-S cluster biogenesis are also possible, such as folding and/or maturation of the target substrates.

Several human diseases are now known to be associated with deficiency of proteins involved in Fe-S cluster biogenesis in mitochondria. For example, frataxin is a key component of the Fe-S cluster assembly machinery, and deficiency of frataxin causes Friedreich ataxia (2, 56). This is an inherited neurodegenerative disease and is characterized by cardiomyopathy and the death of certain neuronal cells, particularly the dorsal root ganglia. Other cell types/tissues are unaffected. Another Fe-S disease, called ISCU myopathy, is caused by low levels of ISCU arising from a splicing mutation of ISCUD11 is essential for both iron-sulfur cluster assembly and maintenance of ISCU (Isu1 in yeast) (57). Unlike Friedreich ataxia, the ISCU myopathy affects primarily skeletal muscles and only rarely the cardiac tissue. The reason for the tissue specificity of these diseases is not known, although tissue-specific splicing differences may contribute, and this has been a challenging issue in the field (3, 9). Alternatively, different tissues may have different requirements for Fe-S clusters, and the corresponding mitochondria may need different levels of frataxin, ISCU, or other components of the cluster machinery. Likewise, nucleotide and/or NADH requirements and the corresponding regulatory steps could also be different from one tissue to the other. These issues can now be addressed, at least partly, by studying Fe-S cluster assembly in mitochondria isolated from different tissues.

Recently, frataxin (Yfh1 in yeast) has been found to form a complex with NFS1 (Nfs1 in yeast), ISD11 (Isd11 in yeast), and ISCU (Isu1 in yeast) (58–61). Using purified proteins, human frataxin has been found to stimulate the cysteine desulfurase activity of the complex (59). In studies of the yeast counterparts using purified proteins and isolated mitochondria, Yfh1 directly interacted with Nfs1, exposing substrate-binding sites, most likely through a conformational change in the enzyme and enhancing the binding of cysteine (46). This unique function of Yfh1 did not require Isu1 or Isd11. In addition, the nucleotides (ATP or GTP), NADH, or iron were not required for frataxin function. Frataxin can compensate for loss of Yfh1 in yeast, and, therefore, the human protein may function in mitochondria via a pathway similar to that in yeast (62, 63). Such a notion can now be directly tested using isolated mammalian mitochondria as described here. This is an important issue because lack of frataxin/Yfh1-mediated stimulatory effects on cysteine desulfurase activity may explain, at least in part, the deficiency of Fe-S clusters in mitochondria associated with frataxin/Yfh1 deficiency (64, 65). The identification of the NFS1-bound persulfide and the tightly coordinated use of the persulfide sulfur with iron for Fe-S cluster synthesis in isolated mammalian mitochondria as described here will be useful in further elucidating the molecular basis of Friedreich ataxia.

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