Published in final edited form as:

Mitochondrion. 2012 September ; 12(5): 539–549. doi:10.1016/j.mito.2012.07.103.

Identification of a Nfs1p-bound persulfide intermediate in Fe-S cluster synthesis by intact mitochondria

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Abstract

Cysteine desulfurases generate a covalent persulfide intermediate from cysteine, and this activated form of sulfur is essential for the synthesis of iron-sulfur (Fe-S) clusters. In yeast mitochondria, there is a complete machinery for Fe-S cluster synthesis, including a cysteine desulfurase, Nfs1p. Here we show that following supplementation of isolated mitochondria with [³⁵S]cysteine, a radiolabeled persulfide could be detected on Nfs1p. The persulfide persisted under conditions that did not permit Fe-S cluster formation, such as nucleotide and/or iron depletion of mitochondria. By contrast, under permissive conditions, the radiolabeled Nfs1p persulfide was greatly reduced and radiolabeled aconitase was formed, indicating transfer of persulfide to downstream Fe-S cluster recipients. Nfs1p in mitochondria was found to be relatively more resistant to inactivation by N-ethylmaleimide (NEM) as compared with a prokaryotic cysteine desulfurase. Mitochondria treated with NEM (1 mM) formed the persulfide on Nfs1p but failed to generate Fe-S clusters on aconitase, likely due to inactivation of downstream recipient(s) of the Nfs1p persulfide. Thus the Nfs1p-bound persulfide as described here represents a precursor *en route* to Fe-S cluster synthesis in mitochondria.

Keywords

Cysteine desulfurase; Nfs1p•Isd11p complex; persulfide; Fe-S clusters; aconitase; yeast

1. Introduction

Iron-sulfur (Fe-S) clusters consist of iron complexed with inorganic sulfur in protein-bound structures capable of varied biochemical reactions. In cells, these clusters are essential cofactors in proteins that mediate diverse functions including electron transfer, oxygen interaction, catalysis, iron regulation, DNA repair, ribosome biogenesis, and tRNA modification among others (Johnson et al., 2005; Rouault and Tong, 2008; Lill, 2009). Fe-S cluster biogenesis is an evolutionarily conserved multi-step biochemical process involving more than a dozen protein components. The process can be broadly understood in terms of

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three phases (Stemmler et al., 2010). In the first phase, sulfur is mobilized from cysteine by an enzyme cysteine desulfurase. In the second phase, sulfur is transferred from the enzyme to scaffold proteins and assembled with iron to form an Fe-S cluster intermediate. In the third phase, mediated by chaperones and glutaredoxins, the Fe-S cluster intermediate is transferred to apoproteins, forming holo and active proteins. In all known cases, the sulfur for Fe-S cluster assembly is derived from cysteine *via* the action of cysteine desulfurases. The biochemical mechanism of these enzymes has been defined by the pioneering work of Dean and co-workers on the NifS ortholog from the nitrogen-fixing prokaryote *Azotobacter vinelandii* (Zheng et al., 1993; Zheng et al., 1994).

Briefly, a conserved lysine forms a Schiff base with the pyridoxal phosphate (PLP) cofactor. The enzyme substrate cysteine interacts with the cofactor, creating a cysteine-PLP ketimine adduct. Nucleophilic attack by the thiolate anion of the active site cysteine on the sulfhydryl group of the substrate-PLP adduct cleaves the C-S bond, resulting in formation of an enzyme-bound persulfide (Enz-S-SH). The substrate cysteine in the PLP adduct is converted to alanine and subsequently released, thereby regenerating PLP for a new catalytic cycle (Zheng et al., 1994; Mihara and Esaki, 2002; Johnson et al., 2005; Selbach et al., 2010). As needed, sulfane sulfur of the persulfide is directly transferred from the enzyme active site to specific recipients such as Isu type scaffolds involved in Fe-S cluster assembly. After sulfur transfer, the active site cysteine and the substrate-binding site of the cysteine desulfurase enzyme are restored to their original state, thus facilitating another cycle of sulfur supply.

In *Saccharomyces cerevisiae*, the cysteine desulfurase is encoded by a single gene *NFS1*. The Nfs1p protein is found mostly in mitochondria (Li et al., 1999), with a small amount functioning in extramitochondrial locations (Nakai et al., 2001). Nfs1p is required for activities of Fe-S cluster proteins (Kispal et al., 1999; Li et al., 1999). Point mutations in the conserved lysine for PLP attachment or in the conserved active site cysteine lead to cell inviability, suggesting that Nfs1p-bound persulfide formation is essential for Fe-S cluster synthesis in mitochondria (Li et al., 1999). In agreement with this notion, a hypomorphic *nfs1* mutant exhibits high cellular iron uptake and accumulates iron in mitochondria. These phenotypic defects are similar to those observed in other yeast mutants that are deficient in mitochondrial Fe-S clusters (Li et al., 1999). Interestingly, some *nfs1* mutants can suppress the lysine auxotrophy of cells lacking the Cu/Zn superoxide dismutase (Strain et al., 1998). Furthermore, Nfs1p appears to be required for thiolation of certain tRNAs involved in mitochondrial protein synthesis (Nakai et al., 2004). Nfs1p associates with a small mitochondrial protein, Isd11p (~11 kDa), and forms a complex (Nfs1p•Isd11p) of ~200 kDa, but the function of Isd11p in mitochondria has not been determined (Adam et al., 2006; Wiedemann et al., 2006). Like the yeast Nfs1p, the major portion of human Nfs1 is also found in mitochondria with a small amount in cytosol/nucleus, and knockdown of the human enzyme by siRNA is lethal likely due to severe deficiency of Fe-S clusters in both mitochondria and cytoplasm (Biederbick et al., 2006; Fosset et al., 2006). Furthermore, the human enzyme also interacts with the homologous Isd11 protein (Shi et al., 2009). However, identification of a bona fide persulfide intermediate on a eukaryotic cysteine desulfurase that can be productively used for Fe-S cluster synthesis in intact mitochondria thus far remained elusive. Here we have resolved this important issue.

Specifically, we present the results of studies using isolated and intact yeast mitochondria supplemented with [³⁵S]cysteine. The [³⁵S] label detected on Nfs1p reflects the cysteine desulfurase activity of the enzyme in mitochondria. Another novel feature of these assays performed with intact mitochondria is that the sulfur label can be detected as newly formed Fe-³⁵S clusters on aconitase in a nucleotide and iron dependent manner (Amutha et al., 2008; Amutha et al., 2009; Pain et al., 2010). Here we show that the signal on Nfs1p

reflecting persulfide formation and the signal on aconitase reflecting new Fe-S cluster synthesis vary reciprocally, indicating a precursor-product relationship.

2. Materials and methods

2.1. Yeast strains, plasmids, growth conditions, and mitochondria isolation

Regulated expression of Nfs1p from the *GAL10* promoter was accomplished by use of the pEMBlyex4i vector carrying the *NFS1* open reading frame (ORF) and integrated at the *URA3* locus of a $\Delta nfs1::HIS3$ yeast strain, as previously described (Li et al., 1999). The Gal-Nfs1p strain was grown at 30°C to mid-logarithmic phase under inducing conditions in YPAR galactose (1% yeast extract, 2% peptone, 100 µg/ml adenine, 2% raffinose and 0.2 % galactose). Cells were harvested, resuspended in YPAR with or without galactose (0.2%), allowed to grow at 30°C for 22 h, and mitochondria were isolated as described (Amutha et al., 2009). Nfs1p persulfide formation or Fe-S cluster biogenesis in mitochondria, isolated from the Gal-Nfs1p strain with the Nfs1p protein expressed, was very similar to those in mitochondria isolated from wild-type strains (D273-10B, BY4741 or YPH499). D273-10B mitochondria were used as wild-type controls. The deletion mutant for *GGC1* carrying the $\Delta ggc1::KanMX$ marker was constructed as described (Lesuisse et al., 2004; Gordon et al., 2006).

2.2. Bacterial expression of proteins

In *S. cerevisiae*, the precursor form of the mitochondrial Nfs1p contains 497 amino acids. Upon import into mitochondria, the N-terminal targeting signal (36 amino acids) of the Nfs1p precursor protein is removed by two different proteases, generating the mature form of the protein (461 amino acids) (Naamati et al., 2009). Unlike in the case for Nfs1p, the accessory protein Isd11p does not appear to contain a cleavable mitochondrial targeting signal (Adam et al., 2006; Wiedemann et al., 2006). The plasmids pST39 and pET3aTr were obtained from Dr. Song Tan (Tan, 2001), and a construct (plasmid 20-38) was generated that contained a T7 promoter driving a polycistronic mRNA for mature Nfs1p with a C-terminal His₆ tag (Nfs1p-His₆) and Isd11p, each with separate ribosome-binding sites. For expression of *A. vinelandii* NifS, the plasmid pT7-7/NifS (pDB551) was obtained from Dr. Dennis R. Dean (Zheng et al., 1993). The ORF was modified with a 5' NdeI restriction site and a 3' His₆ tag followed by a stop codon and BamHI site, generating the plasmid, pT7-7/NifS-His₆.

Proteins were expressed in BL21 (DE3) Codon Plus cells (Stratagene). Cells carrying different plasmids were grown in LB media containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol to OD₆₀₀ of ~0.6. Conditions for expression of proteins were optimized so that the majority of each protein was in soluble form (Amutha and Pain, 2003; Pain et al., 2010). Co-expression of Nfs1p-His₆ and Isd11p was carried out in the presence of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 24 h at 20°C. Expression of *A. vinelandii* NifS-His₆ was induced with 0.5 mM IPTG for 3 h at 37°C. Cells were harvested, and washed with buffer A (50 mM Tris/HCl, pH 8.0, 0.15 M NaCl, 10% glycerol, 1 mM PMSF). Cells were resuspended in buffer A, incubated with lysozyme (50 µg/ml) for 30 min on ice, and then disrupted using a probe sonicator (Branson Sonifier 450). Cell lysates were centrifuged at 12,000 × *g* for 30 min at 4°C, and the supernatant fraction was incubated with Ni-NTA agarose by end-over-end mixing for 3 h at 4°C. The resin was washed with buffer A containing 10 mM imidazole, and bound proteins were eluted with 0.4 M imidazole in buffer A. Purified proteins were stored at -80°C until further use.

2.3. Cysteine desulfurase assays

As necessary, experiments were performed with or without prior depletion of mitochondrial nucleotides. To deplete endogenous nucleotides, isolated and intact mitochondria were preincubated at 30°C for 10 min (Amutha et al., 2008; Amutha et al., 2009; Pain et al., 2010). In a typical 50 µl assay, mitochondria (200 µg of proteins) in HS buffer (20 mM Hepes/KOH, pH 7.5, 0.6 M sorbitol) containing 10 mM Mg(OAc)₂ and 40 mM KOAc were mixed with 10 µCi [³⁵S]cysteine (1075 Ci/mmol). Following incubation at 30°C for different time periods (5–30 min), reaction mixtures were diluted with HS buffer containing 0.15 M NaCl and left on ice for 10 min. Samples were centrifuged at 15,000 × *g* for 10 min at 4°C, and the pellets were analyzed by non-reducing SDS-PAGE followed by autoradiography. In some cases, mitochondria were recovered after incubation with [³⁵S]cysteine as described above and solubilized with the immunoprecipitation (IP) buffer (50 mM Tris/HCl, pH 7.5, 1% Triton X-100, 0.15 M NaCl, 1 mM PMSF). The mitochondrial lysate was centrifuged at 15,000 × *g* for 15 min at 4°C to remove insoluble material, if any, and the supernatant was added to Protein A-Sepharose with prebound anti-Nfs1p antibodies. After incubation at 4°C for 2 h, the beads were washed with the IP buffer, and the bound proteins were eluted with SDS loading buffer containing no DTT. Samples were analyzed by non-reducing SDS-PAGE followed by autoradiography. For some other experiments, mitochondria were pretreated with N-ethylmaleimide (NEM), DTT or α -phenanthroline prior to incubation with [³⁵S]cysteine. These differences are indicated in the corresponding figure legends.

To determine the cysteine desulfurase activity of the purified Nfs1p•Isd11p complex, the reaction mixture contained the protein complex (5–50 ng) in HS buffer containing 0.15 mM PLP, 0.15 M NaCl, and 10 µCi [³⁵S]cysteine in a final volume of 50 µl. Following incubation at 30°C for 15 min, the reaction was stopped and proteins were precipitated with the addition of equal volume of ice-cold 20% trichloroacetic acid (TCA) followed by centrifugation at 15,000 × *g* for 30 min at 4°C. The pellet samples were analyzed by non-reducing SDS-PAGE followed by autoradiography. The cysteine desulfurase activity of purified *A. vinelandii* NifS-His₆ was determined exactly the same way. In some cases, the purified Nfs1p•Isd11p complex or *A. vinelandii* NifS-His₆ was treated with NEM and/or DTT prior to incubation with [³⁵S]cysteine as indicated in the corresponding figure legends.

2.4. Insertion of radiolabeled clusters into aconitase

Isolated mitochondria contain a pool of apoaconitase that can be used as a substrate for Fe-S cluster loading. Assays were performed essentially as described (Amutha et al., 2008; Amutha et al., 2009; Pain et al., 2010; Yoon et al., 2011) with some modifications. Briefly, the reaction mixture (50 µl) included mitochondria (200 µg of proteins) in HS buffer containing 10 mM Mg(OAc)₂ and 40 mM KOAc, with or without a mixture of nucleotides (4 mM ATP, 1 mM GTP, and 2 mM NADH). As necessary, iron was included as ferrous ascorbate to a final concentration of 10 µM. Following addition of [³⁵S]cysteine (10 µCi), samples were incubated at 30°C for 30 min. Reaction mixtures were diluted 20-fold with HS buffer. Mitochondria were recovered by centrifugation, and soluble proteins of mitochondria were analyzed by native PAGE followed by autoradiography, looking for radiolabeling of aconitase (Aco1p).

2.5. Miscellaneous procedures

Radiolabeled protein bands identified as Nfs1p, designated as Nfs1p-S-³⁵SH, or radiolabeled aconitase, indicated as Aco1p [Fe-³⁵S], were quantitated using the Adobe Photoshop CS5 software. Radiolabeling of *A. vinelandii* NifS, indicated as NifS-S-³⁵SH, was also quantitated using the same software. To compare Nfs1p, Isd11p, Aco1p or Mir1p protein levels in various samples, reaction mixtures were analyzed by SDS-PAGE followed by

immunoblotting using the corresponding antibodies. In some cases, samples were also analyzed by Coomassie Blue staining of the SDS gel.

3. Results

3.1. Cysteine desulfurase activity of the purified Nfs1p enzyme complex

Both prokaryotic and eukaryotic cysteine desulfurases contain a critical cysteine residue at their active sites, and the enzymatic cycle of these enzymes involves formation of a covalent persulfide at the active site cysteine (Enz-S-SH). To assess the role of Nfs1p in cysteine desulfurase activity of mitochondria, we began with optimization of a radioactive assay able to detect covalent [³⁵S]-persulfide formation using purified enzymes. For this purpose, Nfs1p-His₆ was co-expressed with its partner protein, Isd11p, in bacteria. Conditions were developed so that the majority of both the proteins remained soluble. Upon Ni-NTA chromatography, Isd11p without a His-tag was co-purified with Nfs1p-His₆ indicating a complex (Nfs1p•Isd11p) formation, and the preparation was more than 90% pure (Fig. 1A, lane 1). The complex formation was further confirmed by sucrose density gradient centrifugation. Following centrifugation, fractions were analyzed by SDS-PAGE followed by immunoblotting using anti-Nfs1p and anti-Isd11p antibodies (data not shown). Nfs1p and Isd11p were found in the same fractions and the molecular mass of the purified complex in the sucrose gradient was determined to be ~200 kDa. The molecular nature and mass of the purified complex were very similar to those of the Nfs1p•Isd11p complex in mitochondria (Adam et al., 2006; Wiedemann et al., 2006). *A. vinelandii* NifS-His₆, a prokaryotic cysteine desulfurase, was purified to homogeneity by Ni-NTA chromatography (Fig. 1A, lane 2).

Purified proteins were incubated with [³⁵S]cysteine and analyzed by non-reducing SDS-PAGE followed by autoradiography (Fig. 1B). For the Nfs1p•Isd11p complex, Nfs1p was radiolabeled indicating covalent persulfide formation on the enzyme (Nfs1p-S-³⁵SH) (lane 1). Persulfides are sensitive to reducing agents, and treatment with DTT prior to SDS-PAGE completely released the persulfide sulfur causing Nfs1p to lose the [³⁵S] label (lane 3). The cysteine desulfurase activity of *A. vinelandii* NifS has been well characterized (Zheng et al., 1993; Zheng et al., 1994) and this prokaryotic enzyme served as control for these experiments. As expected, the NifS enzyme formed the radiolabeled persulfide when incubated with the substrate [³⁵S]cysteine (lane 2) and lost the covalent [³⁵S] signal upon treatment with DTT (lane 4). The radioactive assay as described here reflects a single round of cysteine desulfurase activity, and yet it is highly sensitive and can be easily used to detect persulfide formation on Nfs1p in a concentration-dependent manner in the range of 5-50 ng protein of the Nfs1p•Isd11p complex (Fig. 1C). The activity of this purified Nfs1p•Isd11p complex served as a positive control for studies of the Nfs1p activity in mitochondria (see below).

3.2. Unusual behavior of Nfs1p in SDS gels

As indicated above (Fig. 1B), the persulfide formation on purified enzymes was detected by non-reducing SDS-PAGE followed by autoradiography and not when samples were treated with a reducing agent such as DTT prior to gel electrophoresis. During optimization of these assays, we learned about an unusual behavior of Nfs1p in SDS-gels. Specifically, we found that pretreatment of the purified Nfs1p•Isd11p complex with salt (0.15 M NaCl) was essential for quantitative detection of the Nfs1p protein by non-reducing SDS-PAGE followed by immunoblotting (Fig. 2, Panel 1). In the absence of prior salt treatment, only a small portion of Nfs1p was detected (Panel 1, compare Nfs1p in lanes 1 and 2). Treatment with low (5 mM) or high (80 mM) DTT prior to SDS-PAGE abrogated the need for salt treatment i.e. Nfs1p of the Nfs1p•Isd11p complex was quantitatively visualized by reducing SDS-PAGE with or without treatment with 0.15 M NaCl (Panel 1, lanes 3-6). By contrast,

detection of Isd11p did not require salt and/or DTT treatment of the purified Nfs1p•Isd11p complex prior to SDS gel electrophoresis (Panel 2).

To be able to extend the radioactive persulfide assay to Nfs1p in isolated mitochondria, it was important to make sure that the mitochondrial Nfs1p could be quantitatively detected by the non-reducing gel system. Mitochondrial Nfs1p exhibited the same anomalous behavior as the purified enzyme complex. When mitochondrial proteins were directly analyzed by non-reducing SDS-PAGE and immunoblotting, Nfs1p was virtually undetectable (Fig. 2, Panel 3, lane 1). Pretreatment of mitochondria with salt (0.15M NaCl) in the absence of DTT again allowed detection (Panel 3, lane 2), and addition of DTT did not make any difference (Panel 3, lanes 4 and 6). Treatment with low (5 mM) or high (80 mM) DTT included in the SDS loading buffer allowed detection of mitochondrial Nfs1p without any salt treatment (Panel 3, lanes 3 and 5). Isd11p protein levels served as internal control for these experiments. Unlike in the case of Nfs1p, pretreatment of mitochondria with salt was not necessary for efficient Isd11p detection under non-reducing conditions (Panel 4, lane 1), and addition of DTT in the SDS loading buffer did not make any difference (Panel 4, lanes 3 and 5). Although the reason for the striking effect of salt pretreatment on Nfs1p remains to be determined, this procedure allowed us for the first time to identify the Nfs1p-bound persulfide in isolated and intact mitochondria by non-reducing SDS-PAGE as described below.

3.3. Nfs1p-dependent authentic cysteine desulfurase activity in mitochondria

The *NFS1* gene is essential for yeast cell viability and deletion of the gene is lethal. To assess the role of Nfs1p in cysteine desulfurase activity of mitochondria, we made use of a yeast strain that expresses Nfs1p from the regulated *GAL10* promoter. Protein expression was turned off by shifting from galactoseraffinose (inducing) to raffinose (non-inducing) based media. When switched from inducing to non-inducing growth media, cells stopped synthesizing Nfs1p. The Nfs1p-depleted cells exhibited iron-related phenotypes – high cellular iron uptake, iron accumulation in mitochondria, lack of Fe-S cluster synthesis and loss of aconitase [4Fe-4S] activity (Li et al., 1999; Amutha et al., 2008). These phenotypic defects of Nfs1p-depleted cells were similar to those of a *nfs1* mutant (MA14) with a missense *NFS1* allele (I191S) (Li et al., 1999). MA14 retained Nfs1p expression and yet exhibited iron-related phenotypes because of greatly compromised Nfs1p activity resulting from the hypomorphic mutation. These findings equipped us with two independent strains lacking Nfs1p activity – one that contained no detectable Nfs1p protein (Nfs1p-depleted), the other (MA14) containing Nfs1p mutant protein with greatly reduced activity. The Nfs1p-depleted and MA14 mitochondria were used as negative controls for the experiments described below.

Fe-S cluster synthesis in isolated and intact mitochondria requires addition of nucleotides such as ATP, GTP and NADH (Amutha et al., 2008; Amutha et al., 2009; Pain et al., 2010). However, these nucleotides were not needed for the cysteine desulfurase activity of Nfs1p as judged by persulfide formation by the purified Nfs1p•Isd11p complex in the absence of any added nucleotides (Fig. 1B). Nucleotides therefore appear to be required for the downstream events such as Fe-S cluster synthesis on scaffold proteins and/or their transfer to apoproteins. To identify the Nfs1p-bound persulfide, our strategy was to prevent transfer of the persulfide sulfur to downstream recipients, thereby “trapping” the persulfide on the enzyme. We therefore performed experiments with mitochondria after depletion of endogenous nucleotides. Briefly, isolated and intact mitochondria were depleted for endogenous nucleotides, incubated with [³⁵S]cysteine, and analyzed by non-reducing SDS-PAGE followed by autoradiography (Fig. 3A, top panel). Identical samples were also evaluated for Nfs1p and Isd11p proteins by immunoblotting (Fig. 3A, middle and bottom panels, respectively), thereby serving as loading controls.

A major radiolabeled protein (51 kDa) was detected in wild-type (WT) mitochondria (Fig. 3A, top panel, lane 2). Most likely, the radiolabeling was due to the covalent [^{35}S]-persulfide formed on Nfs1p (Nfs1p-S- ^{35}SH) in mitochondria. The 51 kDa radiolabeled band in WT mitochondria did not contain any His₆ tag and migrated close to but slightly faster than the radiolabeled Nfs1p containing a His₆ tag that was generated when purified Nfs1p•Isd11p complex was incubated with [^{35}S]cysteine (top panel, lane 1). Furthermore, the ~51 kDa radiolabeled band was absent in both Nfs1p-depleted (Nfs1p with a downward arrowhead) and MA14 mitochondria (top panel, lanes 3 and 4, respectively). Nfs1p-depleted mitochondria did not contain any detectable Nfs1p protein and MA14 mitochondria contained an inactive form of Nfs1p (middle panel). However, both of these mutant mitochondria contained the Nfs1p partner protein, Isd11p, at wild-type levels (bottom panel), thereby serving as loading control. The overall protein patterns of WT, Nfs1p-depleted and MA14 mitochondria were also comparable as shown by Coomassie Blue staining of a gel (Supplementary Fig. S1, top panel). The corresponding immunoblot (Fig. S1, bottom panel) showing similar protein levels of an unrelated mitochondrial marker protein, Mir1p, served as additional loading control.

Treatment with a reducing agent such as DTT prior to SDS-PAGE released the persulfide sulfur, and radiolabeled Nfs1p was no longer detected in WT mitochondria (Fig. 3A, top panel, lane 5). These results also show that [^{35}S]cysteine was not incorporated into the protein backbone as an amino acid forming a covalent peptide bond, because the radiolabel was released upon DTT treatment. Such a notion was further confirmed by the observation that radiolabeling of Nfs1p was unaffected by the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis, indicating that mitochondrial protein synthesis did not contribute to radiolabeling of the protein (Fig. 3B, top panel). These results are consistent with our earlier observation that blocking mitochondrial protein synthesis with the addition of chloramphenicol did not interfere with Fe-S cluster synthesis in isolated wild-type mitochondria (Amutha et al., 2008). Finally, the identity of the radiolabeled 51 kDa protein as Nfs1p was further substantiated by immunoprecipitation (IP) of the protein from wild-type mitochondria. Nfs1p-depleted and MA14 mitochondria incubated with [^{35}S]cysteine were included as controls (Fig. 3C). These results confirm that the radiolabeled 51 kDa protein in wild-type mitochondria is indeed Nfs1p and that the [^{35}S] radiolabel associated with Nfs1p represents [^{35}S]-persulfide formation on the enzyme.

Several other radiolabeled proteins were detected regardless of the presence or absence of Nfs1p in mitochondria (Fig. 3A, top panel, lanes 2-4), and hence they were considered to be background bands. A redundant cysteine desulfurase gene/protein might explain such results. However, no homolog exists in the yeast genome, and mitochondria from the Nfs1p-depleted and MA14 cells were deficient in activities for Fe-S cluster proteins such as aconitase and succinate dehydrogenase (Li et al., 1999; Amutha et al., 2008). The Nfs1p-independent radiolabeling of the background bands in mitochondria could be due to cysteinylolation or H₂S formation and protein S-sulfhydration of these proteins (Hochgräfe et al., 2007; Mustafa et al., 2009; Chouchani et al., 2011). Such a notion is consistent with the observation that upon treatment with DTT, radiolabeling of the background bands was abrogated (Fig. 3A, top panel, lanes 5-7). In any case, these results show that the [^{35}S]-persulfide formed on Nfs1p by isolated mitochondria can be identified with certainty and distinguished from other radiolabeled proteins.

3.4. Nfs1p-dependent persulfide formation – kinetics and NEM sensitivity

As expected, persulfide formation on Nfs1p in wild-type mitochondria appeared in a time-dependent manner (Fig. 4A, top panel, lanes 1-4). Cysteine desulfurases generate persulfide at the active site cysteine residue, and modification of the active site cysteine by an alkylating agent such as NEM inactivates the enzyme (Zheng et al., 1993). Unlike cysteine

desulfurases, the eukaryote-specific Isd11p does not contain any cysteine residues. To further characterize the Nfs1p-associated persulfide, intact mitochondria were treated with increasing concentrations of NEM, and then excess NEM was neutralized with DTT. Mitochondria were washed to remove both NEM and DTT and subsequently incubated with [³⁵S]cysteine. As shown in Fig. 4B (top panel), persulfide formation on Nfs1p was inhibited by prior NEM treatment in a concentration-dependent manner. After treatment with 0.5 mM NEM, the enzyme still retained 100% activity and complete inhibition was observed at 4 mM NEM. These results were surprising, considering that prokaryotic cysteine desulfurases are highly sensitive to inactivation by alkylating agents such as NEM. For example, the *A. vinelandii* NifS was almost completely inhibited after treatment with as low as 0.1 mM NEM (Fig. 4C).

In mitochondria, Nfs1p might exist in different forms. For example, the active site cysteine of the enzyme in mitochondria might already contain a bound persulfide (unlabeled). Likewise, the active site might be protected through modifications, such as S-cysteinylation or S-glutathionylation. In both of these cases, the Nfs1p active site cysteine would not be available for NEM modification and subsequent DTT treatment would convert the active site cysteine back to its free -SH form, thereby allowing it to interact with the added substrate [³⁵S]cysteine and to form a radiolabeled persulfide. To test these possibilities, mitochondria were first treated with DTT to convert any modified active site cysteines of Nfs1p back to free thiols. After washing to remove DTT, mitochondria were treated with NEM and subsequently assayed exactly as in Fig. 4B. There was no significant change in NEM-sensitivity of Nfs1p (Supplementary Fig. S2A, top panel), implying that the active site cysteine of the enzyme in isolated mitochondria is unlikely to be blocked. Any significant modification/protection of the active site cysteine through S-cysteinylation or S-glutathionylation seems unlikely. This notion was further substantiated by experiments with the bacterially expressed and purified Nfs1p•Isd11p complex. The purified complex was treated with increasing concentrations of NEM, and then excess NEM was neutralized with DTT. After removal of both NEM and DTT, the Nfs1p•Isd11p complex was incubated with [³⁵S]cysteine. The persulfide formation on Nfs1p was practically unaffected by 0.5 mM NEM pretreatment (Fig. 4D, top panel). Furthermore, treatment of the complex first with DTT (prior to NEM treatment) did not alter the NEM sensitivity of Nfs1p of the purified complex (Supplementary Fig. S2B, top panel). In summary, Nfs1p in mitochondria (Figs. 4B and S2A) and the purified Nfs1p•Isd11p complex (Figs. 4D and S2B) behaved very similarly with respect to the active site cysteine availability and inactivation by NEM.

3.5 Nucleotide-dependent chase of the Nfs1p persulfide for Fe-S cluster synthesis

In the physiologic setting in mitochondria, the cysteine desulfurase most likely undergoes repeated cycles of activity: binding cysteine, forming persulfide, and transferring persulfide to recipients. We therefore asked if the persulfide formed on Nfs1p in our assays represents a productive intermediate for Fe-S cluster synthesis and thus reflects a balance between persulfide formation and persulfide transfer. For this purpose, experiments were performed under conditions that “trap” the [³⁵S] label at the persulfide level (nucleotide depletion and no iron addition) or under conditions that promote cluster synthesis. Nucleotides (ATP, GTP and NADH) are unnecessary for cysteine desulfurase activity (Figs. 1B and 3), but are required for Fe-S cluster formation in mitochondria (Amutha et al., 2008; Amutha et al., 2009; Pain et al., 2010). In nucleotide-depleted mitochondria, the cysteine desulfurase persulfide should be formed and arrested, because Fe-S cluster synthesis is blocked. Reciprocally, in mitochondria with optimal nucleotides, the persulfide should be formed and transferred as part of the Fe-S cluster synthesis process, and therefore the steady state persulfide signal should be reduced. These predictions were borne out in wild-type

mitochondria (Fig. 5A). Nfs1p-depleted and MA14 mitochondria served as negative controls.

Briefly, isolated mitochondria were depleted of endogenous nucleotides and then incubated with [35 S]cysteine in the absence or presence of added nucleotides (ATP, GTP and NADH) and ferrous ascorbate as an iron source. One set of samples was analyzed for Nfs1p-S- 35 SH formation and Nfs1p protein levels (Fig. 5A, left panel). Another set of identical samples was evaluated for Fe- 35 S cluster loading of aconitase and the corresponding aconitase (Aco1p) protein levels (Fig. 5A, right panel). The latter was based on our earlier findings that isolated wild-type mitochondria contain a pool of apoaconitase and a nucleotide-dependent machinery capable of forming new clusters and inserting them into this endogenous apoprotein pool (Amutha et al., 2008; Amutha et al., 2009; Pain et al., 2010). In wild-type mitochondria, addition of nucleotides and iron led to greatly reduced [35 S]-persulfide detection (Fig. 5A, left panel, compare lanes 1 and 2) with concomitant increase in Fe-S cluster formation as judged by enhanced radiolabeling of aconitase (right panel, compare lanes 1 and 2). The reciprocal relationship suggests that in the presence of added nucleotides and iron supplementation, the Nfs1p-associated persulfide was utilized for Fe- 35 S cluster synthesis of aconitase. To further validate this notion, similar experiments were performed with $\Delta ggc1$ mitochondria serving as control. Ggc1p is a GTP/GDP carrier protein in the mitochondrial inner membrane that allows exchange of cytosolic GTP for matrix GDP (Voza et al., 2004). This exchange process does not occur in $\Delta ggc1$ mitochondria, and these mutant mitochondria are deficient in Fe-S cluster synthesis because of inadequate levels of matrix GTP (Amutha et al., 2008). The $\Delta ggc1$ mutant mitochondria contained the Nfs1p protein at wild-type level, and were able to form the [35 S]-persulfide on Nfs1p in the absence of added nucleotides and iron, as in WT mitochondria. However, unlike in the case of WT mitochondria, the persulfide level in $\Delta ggc1$ mitochondria remained unchanged with the addition of nucleotides (including GTP) and iron (Fig. 5A, left panel, lanes 3 and 4). As expected, practically no radiolabeled aconitase was detected in $\Delta ggc1$ mitochondria even though the mutant mitochondria contained wild-type level of aconitase protein (right panel, lanes 3 and 4). The inability of $\Delta ggc1$ mitochondria to form new Fe-S clusters was due to failure of these mutant mitochondria to transport added GTP into the matrix. In the absence of adequate levels of GTP in the matrix, Fe-S clusters were not synthesized (Amutha et al., 2008). These results strongly suggest that while nucleotides are not required for persulfide formation, they are essential for utilization of the Nfs1p-bound persulfide for Fe-S cluster synthesis in mitochondria.

To provide further evidence for nucleotide-dependent chase of the persulfide for Fe-S cluster synthesis, we took advantage of the observation that Nfs1p in mitochondria was quite resistant to inactivation by 1 mM NEM (Fig. 4B). Unlike in the case of Nfs1p, the critical cysteine residues of the downstream persulfide sulfur recipients in mitochondria, such as the scaffold proteins Isu1p/Isu2p and/or aconitase, might be more sensitive to inactivation by NEM. In that case, the Nfs1p-bound persulfide, generated after pretreatment of mitochondria with low concentrations of NEM, might not be utilized for Fe-S cluster synthesis even in the presence of added nucleotides. To test this possibility, nucleotide-depleted wild-type mitochondria were treated with NEM (1 mM) and then excess NEM was neutralized with DTT. Following washing to remove both NEM and DTT, mitochondria were incubated with [35 S]cysteine in the absence or presence of added nucleotides and ferrous ascorbate. With no prior NEM treatment, mitochondria were able to form Nfs1p-S- 35 SH, and the [35 S]-persulfide level was greatly reduced with the addition of nucleotides and iron (Fig. 5B, top panel, compare lanes 1 and 2). By contrast, NEM-treated mitochondria were capable of forming the persulfide but the persulfide remained “trapped” on Nfs1p even after addition of nucleotides and iron (Fig. 5B, compare lanes 3 and 4), likely due to inactivation of downstream recipient(s) of the Nfs1p persulfide by NEM. In fact,

NEM-treated wild-type mitochondria completely failed to synthesize Fe-³⁵S cluster of aconitase under identical conditions (Fig. 5C, top panel, lanes 3 and 4). Efficient radiolabeling of aconitase in NEM-untreated mitochondria in the presence of added nucleotides and iron served as positive control (Fig. 5C, top panel, lane 2). These results for the first time identify a productive persulfide intermediate on Nfs1p that can be utilized for Fe-S cluster synthesis in isolated and intact mitochondria.

3.6 Iron-dependent use of the Nfs1p persulfide

Fe-S cluster synthesis requires both iron and sulfur, and the experiments for Fig. 5 as discussed above were performed with or without added iron together with nucleotides. We therefore tested if the nucleotide-dependent utilization of the Nfs1p-associated persulfide for cluster synthesis is dependent on iron availability. For this purpose, a two-step assay was developed as follows. Intact mitochondria were incubated with or without α -phenanthroline, a membrane permeable metal chelator (1st step). Following washing, mitochondria were supplemented with [³⁵S]cysteine, and incubated with or without added nucleotides and/or ferrous ascorbate as an iron source (2nd step). Identical samples were analyzed for Nfs1p-S-³⁵SH (Fig. 6, left panel) and Aco1p [Fe-³⁵S] (Fig. 6, right panel). Nfs1p and Aco1p protein levels served as corresponding loading controls.

In the absence of prior α -phenanthroline treatment, the [³⁵S]-persulfide level was significantly reduced with the addition of nucleotides alone and the decrease was even greater when both nucleotides and iron were included (Fig. 6, left panel, lanes 1-3). Most likely, the decrease in Nfs1p-S-³⁵SH levels was due to concomitant increase in Fe-³⁵S cluster synthesis of aconitase in the corresponding samples (Fig. 6, right panel, lanes 1-3). These results are consistent with our previous results on Fe-S cluster synthesis under similar conditions. Specifically, we found that isolated wild-type mitochondria contain a pool of endogenous iron, which can be used for Fe-S cluster synthesis in a nucleotide-dependent manner and that the process is stimulated with the addition of reduced iron (Amutha et al., 2008; Pain et al., 2010). When mitochondria were pretreated with α -phenanthroline, the [³⁵S]-persulfide mostly persisted even after addition of nucleotides as long as no iron was added (left panel, lanes 4 and 5) and no radiolabeling of aconitase was detected (right panel, lanes 4 and 5). When iron was included together with nucleotides, the persulfide was no longer detected (left panel, lane 6) and aconitase became radiolabeled (right panel, lane 6). The chelator α -phenanthroline, by virtue of its ability to cross the mitochondrial inner membrane, likely bound and immobilized iron from an endogenous pool (Amutha et al., 2008). In the absence of available iron, Fe-S cluster synthesis did not occur even in the presence of added nucleotides and the Nfs1p-associated persulfide was therefore not utilized and remained “trapped”. Addition of iron together with nucleotides allowed new Fe-³⁵S clusters to be made and the persulfide was used in the process. Thus utilization of the Nfs1p-associated persulfide for Fe-S cluster synthesis in these assays with isolated and intact mitochondria is both nucleotide and iron dependent.

4. Discussion

In *S. cerevisiae*, *NFS1* is the sole gene coding for a cysteine desulfurase, and apparently the corresponding protein Nfs1p is the only source of sulfur for Fe-S cluster synthesis (Li et al., 1999). Thus not surprisingly *NFS1* is an essential gene. The major portion of Nfs1p protein is found in mitochondria where it participates in Fe-S cluster assembly and other processes. During the cysteine desulfurase enzymatic cycle, a persulfide is formed at the active site cysteine of Nfs1p and then sulfane sulfur (S⁰) is delivered to recipient proteins involved in Fe-S cluster synthesis or thio modification of tRNAs (Mueller, 2006; Stemmler et al., 2010). Here we report on a new and sensitive radiolabeling assay for the Nfs1p persulfide in isolated and intact mitochondria. Following incubation of mitochondria with [³⁵S]cysteine, a

radiolabeled persulfide reflecting the Nfs1p activity (Nfs1p-S-³⁵SH) was detected by non-reducing SDS-PAGE and autoradiography, and its characteristics reflected the enzyme activity of the mitochondrial cysteine desulfurase. The appearance of the Nfs1p-bound persulfide depended not only on the Nfs1p protein but also on the Nfs1p activity (Figs. 3 A & C) and occurred in a time-dependent manner (Fig. 4A). The Nfs1p-bound persulfide formed in mitochondria (Fig. 3A), like its counterpart generated by the bacterially expressed and purified Nfs1p•Isd11p enzyme complex (Fig. 1B), was sensitive to DTT treatment. Likewise, an alkylating agent, such as NEM, inhibited the cysteine desulfurase activity of both the mitochondrial Nfs1p (Figs. 4B and S2A) and the purified enzyme complex (Figs. 4D and S2B) to similar extents in a dose-dependent manner. Most importantly, the Nfs1p-bound persulfide generated in mitochondria could be chased from the enzyme and subsequently utilized for Fe-S cluster synthesis under optimal metabolic conditions of mitochondria (Figs. 5 and 6). Thus for the first time the Nfs1p-associated persulfide as reported here can be seen to function as a true and productive biochemical intermediate for Fe-S cluster biogenesis in isolated and intact mitochondria.

Even though Nfs1p was identified as a cysteine desulfurase in mitochondria more than a decade ago (Kispal et al., 1999; Li et al., 1999), the Nfs1p-bound persulfide was not previously identified in isolated and intact mitochondria. The major reasons for this gap include: the unusual behavior of the Nfs1p protein in non-reducing SDS-gels, lack of sensitive and reproducible persulfide-forming assays, difficulty in distinguishing the real Nfs1p activity from background activities in mitochondria, and the complexity in establishing the Nfs1p-bound persulfide as a bona fide intermediate for Fe-S cluster synthesis. Here we have resolved all these issues to a great extent. A previously used assay for the cysteine desulfurase activity in mitochondria involved incubation of mitochondrial lysate with the amino acid cysteine (unlabeled) under reducing conditions and colorimetric detection of continuous persulfide formation and release of persulfide sulfur as sulfide (Adam et al., 2006; Wiedemann et al., 2006). An earlier study used the same assay to determine the activity of bacterially expressed Nfs1p alone (Mühlenhoff et al., 2004), although at the time Nfs1p partner protein, Isd11p, was not yet identified. We found that the spectrophotometric assay did not detect any significant activity of 5 µg of purified Nfs1p alone with no Isd11p; it required a minimum of 2-5 µg of the purified Nfs1p•Isd11p complex or *A. vinelandii* NifS (data not shown). Such an insensitive assay therefore could not be reliably used for detection of cysteine desulfurase activity in mitochondria in which Nfs1p is present at less than 0.01%. In fact, experiments with Nfs1p-depleted mitochondria yielded completely unexpected results. Although Nfs1p is the only known cysteine desulfurase protein in mitochondria, mitochondria with no detectable Nfs1p retained strong sulfide-generating activity, which most likely represents background activity (data not shown). The basis for the Nfs1p-independent sulfur release from cysteine has not been ascertained, but it might be due to cystathionine β-synthase or similar enzymes that release H₂S and perform transsulfuration reactions (Mustafa et al., 2009). By contrast with the colorimetric assays, the radioactive assay as described here was at least 50-100 times more sensitive, and [³⁵S]-persulfide formation was easily detected with as little as 5-50 ng of the purified Nfs1p•Isd11p complex (Fig. 1C) or 50-200 µg of total mitochondrial proteins (data not shown) in a concentration-dependent manner. Identification of the Nfs1p-bound persulfide was confirmed by immunoprecipitation experiments (Fig. 3C). Furthermore, the persulfide formation on Nfs1p, as expected, was strictly dependent on both Nfs1p protein and activity (Figs. 3 A & C). For example, no Nfs1p-S-³⁵SH was detected in Nfs1p-depleted or MA14 mitochondria. Whereas the former did not contain any detectable Nfs1p protein, the latter contained an inactive form of Nfs1p. Thus the use of two different positive controls (persulfide formed by the purified Nfs1p•Isd11p complex and immunoprecipitation of the Nfs1p-bound persulfide from mitochondria) and two different negative controls (Nfs1p-depleted or MA14 mitochondria incubated with [³⁵S]cysteine) allowed us to identify

the Nfs1p-bound persulfide in mitochondria and to distinguish it from other background radiolabeled proteins with confidence. These experiments were possible because we were able to determine conditions that permitted quantitative detection of the Nfs1p protein by SDS-PAGE but did not interfere with the persulfide on Nfs1p. Specifically, treatment of the Nfs1p•Isd11p complex or mitochondria with salt (0.15 M NaCl) was critical to bypass the requirement for a reducing agent such as DTT in the SDS loading buffer for gel analysis (Fig. 2).

A key feature of the Nfs1p persulfide assay described here is that it could be performed with intact mitochondria, thereby allowing manipulations of the organelle milieu that influence both the cysteine desulfurase and Fe-S cluster-forming activities. The mitochondrial Nfs1p persulfide, reflecting the cysteine desulfurase activity, exhibited features of a biochemical intermediate. Fe-S cluster assembly in mitochondria occurs in defined stages. First, the Nfs1p persulfide must be formed. Then the persulfide is transferred to the scaffold proteins (Isu1p/Isu2p) and assembled with iron to form an Fe-S cluster intermediate. Finally, the Fe-S cluster intermediate is delivered to apoproteins in a chaperone and glutaredoxin dependent step (Lill, 2009; Stemmler et al., 2010). Nucleotides such as ATP, GTP and NAD(P)H are independently required for Fe-S cluster formation in mitochondria (Amutha et al., 2008; Amutha et al., 2009; Pain et al., 2010). NAD(P)H is needed for ferredoxin reductase (Arh1p)-ferredoxin (Yah1p) redox couple and reducing equivalents are needed for sulfur or iron transfer to the Fe-S scaffold intermediate (Li et al., 2001; Mühlenhoff et al., 2002; Pain et al., 2010). GTP is needed at an early step of Fe-S cluster intermediate formation on scaffold proteins, although the biochemical target of the GTP requirement has not yet been identified (Amutha et al., 2008). ATP is likely needed for the Hsp70 chaperone Ssq1p, functioning in transfer of the cluster intermediates to apoproteins (Knight et al., 1998; Dutkiewicz et al., 2003; Mühlenhoff et al., 2003; Amutha et al., 2008). However, none of the above nucleotides are needed for the cysteine desulfurase activity of Nfs1p. Thus nucleotide depletion of intact mitochondria that were subsequently incubated with [³⁵S]cysteine allowed formation of the Nfs1p persulfide but not its release, because the downstream steps involved in persulfide utilization were blocked. Addition of nucleotides (and iron) greatly reduced the level of [³⁵S]-persulfide on Nfs1p, with concomitant synthesis and insertion of Fe-³⁵S clusters into aconitase (Fig. 5A). Thus the Nfs1p-bound persulfide represents a genuine biochemical intermediate *en route* to Fe-S cluster synthesis in mitochondria. Further supporting evidence for this conclusion came from completely independent experiments as follows.

Like in the case of nucleotide-depleted wild-type mitochondria, mitochondria isolated from a specific deletion strain, $\Delta ggc1$, lacking the mitochondrial GTP/GDP carrier presented a comparable biochemical phenotype in mitochondria i.e. persulfide formation but lack of release (Fig. 5A). However, unlike in the case of wild-type mitochondria, addition of nucleotides to intact $\Delta ggc1$ mitochondria failed to release the persulfide from Nfs1p and no Aco1p [Fe-³⁵S] was formed because GTP (one of the components of the nucleotide mixture) was unable to enter the matrix to support Fe-S cluster synthesis. In another experiment, wild-type mitochondria treated with NEM (1 mM) was able to form the persulfide on Nfs1p but failed to release and utilize the persulfide for Fe-S cluster synthesis even after nucleotide and iron supplementation (Figs. 5 B & C). Most likely, the critical cysteine residues of downstream recipients (e.g. the scaffold proteins Isu1p/Isu2p) of the persulfide sulfur from Nfs1p were inactivated by NEM under these conditions, leaving the persulfide “trapped” on Nfs1p. An interesting question is why the active site cysteine of Nfs1p in mitochondria or in the purified Nfs1p•Isd11p complex is relatively more resistant to inactivation by NEM when compared with the NEM-sensitivity of a prokaryotic cysteine desulfurase like *A. vinelandii* NifS. Whereas the prokaryotic enzyme was almost completely inactivated by 0.1 mM NEM (Fig. 4C), the eukaryotic enzyme was not at all affected at five times higher concentration of

NEM (Figs. 4 B & D, and S2). The Nfs1p-interacting protein Isd11p is conserved from yeast to humans, but no homolog is found in prokaryotes. Unlike *A. vinelandii* NifS, Nfs1p in mitochondria might need to be activated through an interaction with Isd11p while protecting the active site cysteine of the enzyme. This issue will be addressed in a publication elsewhere.

Similar to the nucleotide requirement, iron, an essential constituent of Fe-S clusters, was also required for the transfer/utilization of Nfs1p persulfide in mitochondria. Thus intact mitochondria, treated with a membrane-permeable chelator to remove iron and subsequently incubated with [³⁵S]cysteine, were able to form the Nfs1p persulfide but unable to efficiently release it for subsequent Fe-S cluster synthesis even in the presence of added nucleotides. Only when both nucleotides and iron were added, the persulfide was completely released and utilized for Fe-S cluster synthesis with the generation of Aco1p [Fe-³⁵S] (Fig. 6). Sulfur is a vital element for cell viability and the persulfide, an activated form of sulfur, is essential for the synthesis of not only Fe-S clusters but also other cofactors such as biotin, lipoic acid, and thiamin (Beinert, 2000; Kessler, 2006; Mueller, 2006). The persulfide is also required for the thio-modification of tRNAs. Our studies as presented here will be useful towards understanding the mechanistic details of Fe-S cluster biogenesis in mitochondria and other metabolic processes that require the persulfide sulfur.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Dennis R. Dean for the plasmid pT7-7/NifS and Dr. Song Tan for the plasmids pST39 and pET3aTr. We also thank Boominathan Amutha for valuable contribution in the early stage of this work. This work was supported in part by National Institutes of Health Grant GM087965 from NIGMS (to D.P. and A.D.), Grant AG030504 from NIA (to D.P.), and Grant R37DK053953 (to A.D.). This work was also supported by American Heart Association Grant 09GRNT2260364 (to D.P.).

Abbreviations

PLP	pyridoxal phosphate
NEM	N-ethylmaleimide

References

- Adam AC, Bornhövd C, Prokisch H, Neupert W, Hell K. The Nfs1 interacting protein Isd11 has an essential role in Fe/S cluster biogenesis in mitochondria. *EMBO J.* 2006; 25:174–183. [PubMed: 16341090]
- Amutha B, Gordon DM, Dancis A, Pain D. Nucleotide-dependent iron-sulfur cluster biogenesis of endogenous and imported apoproteins in isolated intact mitochondria. *Methods Enzymol.* 2009; 456:247–266. [PubMed: 19348893]
- Amutha B, Gordon DM, Gu Y, Lyver ER, Dancis A, Pain D. GTP is required for iron-sulfur cluster biogenesis in mitochondria. *J. Biol. Chem.* 2008; 283:1362–1371. [PubMed: 18029354]
- Amutha B, Pain D. Nucleoside diphosphate kinase of *Saccharomyces cerevisiae*, Ynk1p: localization to the mitochondrial intermembrane space. *Biochem. J.* 2003; 370:805–815. [PubMed: 12472466]
- Beinert H. A tribute to sulfur. *Eur. J. Biochem.* 2000; 267:5657–5664. [PubMed: 10971575]
- Biederbick A, Stehling O, Rösser R, Niggemeyer B, Nakai Y, Elsässer H-P, Lill R. Role of human mitochondrial Nfs1 in cytosolic iron-sulfur protein biogenesis and iron regulation. *Mol. Cell. Biol.* 2006; 26:5675–5687. [PubMed: 16847322]

- Chouchani ET, James AM, Fearnley IM, Lilley KS, Murphy MP. Proteomic approaches to the characterization of protein thiol modification. *Curr. Opin. Chem. Biol.* 2011; 15:120–128. [PubMed: 21130020]
- Dutkiewicz R, Schilke B, Knieszner H, Walter W, Craig EA, Marszalek J. Ssq1, a mitochondrial Hsp70 involved in iron-sulfur (Fe/S) center biogenesis: Similarities and differences from its bacterial counterpart. *J. Biol. Chem.* 2003; 278:29719–29727. [PubMed: 12756240]
- Fosset C, Chauveau M-J, Guillon B, Canal F, Drapier J-C, Bouton C. RNA silencing of mitochondrial m-Nfs1 reduces Fe-S enzyme activity both in mitochondria and cytosol of mammalian cells. *J. Biol. Chem.* 2006; 281:25398–25406. [PubMed: 16787928]
- Gordon DM, Lyver ER, Lesuisse E, Dancis A, Pain D. GTP in the mitochondrial matrix plays a crucial role in organellar iron homeostasis. *Biochem. J.* 2006; 400:163–168. [PubMed: 16842238]
- Hochgräfe F, Mostertz J, Pöther D-C, Becher D, Helmann JD, Hecker M. S-Cysteinylation is a general mechanism for thiol protection of *Bacillus subtilis* proteins after oxidative stress. *J. Biol. Chem.* 2007; 282:25981–25985. [PubMed: 17611193]
- Johnson DC, Dean DR, Smith AD, Johnson MK. Structure, function, and formation of biological iron-sulfur clusters. *Annu. Rev. Biochem.* 2005; 74:247–281. [PubMed: 15952888]
- Kessler D. Enzymatic activation of sulfur for incorporation into biomolecules in prokaryotes. *FEMS Microbiol. Rev.* 2006; 30:825–840. [PubMed: 17064282]
- Kispal G, Csere P, Prohl C, Lill R. The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. *EMBO J.* 1999; 18:3981–3989. [PubMed: 10406803]
- Knight SAB, Sepuri NBV, Pain D, Dancis A. Mt-Hsp70 homolog, Ssc2p, required for maturation of yeast frataxin and mitochondrial iron homeostasis. *J. Biol. Chem.* 1998; 273:18389–18393. [PubMed: 9660806]
- Lesuisse E, Lyver ER, Knight SAB, Dancis A. Role of *YHMI*, encoding a mitochondrial carrier protein, in iron distribution of yeast. *Biochem. J.* 2004; 378:599–607. [PubMed: 14629196]
- Li J, Kogan M, Knight SAB, Pain D, Dancis A. Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution. *J. Biol. Chem.* 1999; 274:33025–33034. [PubMed: 10551871]
- Li J, Saxena S, Pain D, Dancis A. Adrenodoxin reductase homolog (Arh1p) of yeast mitochondria required for iron homeostasis. *J. Biol. Chem.* 2001; 276:1503–1509. [PubMed: 11035018]
- Lill R. Function and biogenesis of iron-sulphur proteins. *Nature.* 2009; 460:831–838. [PubMed: 19675643]
- Mihara H, Esaki N. Bacterial cysteine desulfurases: their function and mechanisms. *Appl. Microbiol. Biotechnol.* 2002; 60:12–23. [PubMed: 12382038]
- Mueller EG. Trafficking in persulfides: delivering sulfur in biosynthetic pathways. *Nat. Chem. Biol.* 2006; 2:185–194. [PubMed: 16547481]
- Mühlenhoff U, Balk J, Richhardt N, Kaiser JT, Sipos K, Kispal G, Lill R. Functional characterization of the eukaryotic cysteine desulfurase Nfs1p from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 2004; 279:36906–36915. [PubMed: 15220327]
- Mühlenhoff U, Gerber J, Richhardt N, Lill R. Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p. *EMBO J.* 2003; 22:4815–4825. [PubMed: 12970193]
- Mühlenhoff U, Richhardt N, Gerber J, Lill R. Characterization of iron-sulfur protein assembly in isolated mitochondria: A requirement for ATP, NADH, and reduced iron. *J. Biol. Chem.* 2002; 277:29810–29816. [PubMed: 12065597]
- Mustafa AK, Gadalla MM, Sen N, Kim S, Mu W, Gazi SK, Barrow RK, Yang G, Wang R, Snyder SH. H₂S signals through protein S-sulphydration. *Sci. Signal.* 2009; 2:ra72. [PubMed: 19903941]
- Naamati A, Regev-Rudzki N, Galperin S, Lill R, Pines O. Dual targeting of Nfs1 and discovery of its novel processing enzyme, Icp55. *J. Biol. Chem.* 2009; 284:30200–30208. [PubMed: 19720832]
- Nakai Y, Nakai M, Hayashi H, Kagamiyama H. Nuclear localization of yeast Nfs1p is required for cell survival. *J. Biol. Chem.* 2001; 276:8314–8320. [PubMed: 11110795]
- Nakai Y, Umeda N, Suzuki T, Nakai M, Hayashi H, Watanabe K, Kagamiyama H. Yeast Nfs1p is involved in thio-modification of mitochondrial and cytoplasmic tRNAs. *J. Biol. Chem.* 2004; 279:12363–12368. [PubMed: 14722066]

- Pain J, Balamurali MM, Dancis A, Pain D. Mitochondrial NADH kinase, Pos5p, is required for efficient iron-sulfur cluster biogenesis in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 2010; 285:39409–39424. [PubMed: 20889970]
- Rouault TA, Tong WH. Iron-sulfur cluster biogenesis and human disease. *Trends Genet.* 2008; 24:398–407. [PubMed: 18606475]
- Selbach B, Earles E, Dos Santos PC. Kinetic analysis of the bisubstrate cysteine desulfurase SufS from *Bacillus subtilis*. *Biochemistry.* 2010; 49:8794–8802. [PubMed: 20822158]
- Shi Y, Ghosh MC, Tong W-H, Rouault TA. Human ISD11 is essential for both iron-sulfur cluster assembly and maintenance of normal cellular iron homeostasis. *Hum. Mol. Genet.* 2009; 18:3014–3025. [PubMed: 19454487]
- Stemmler TL, Lesuisse E, Pain D, Dancis A. Frataxin and mitochondrial Fe-S cluster Biogenesis. *J. Biol. Chem.* 2010; 285:26737–26743. [PubMed: 20522547]
- Strain J, Lorenz CR, Bode J, Garland S, Smolen GA, Ta DT, Vickery LE, Culotta VC. Suppressors of superoxide dismutase (*SOD1*) deficiency in *Saccharomyces cerevisiae*. Identification of proteins predicted to mediate iron-sulfur cluster assembly. *J. Biol. Chem.* 1998; 273:31138–31144. [PubMed: 9813017]
- Tan S. A modular polycistronic expression system for overexpressing protein complexes in *Escherichia coli*. *Protein Expr. Purif.* 2001; 21:224–234. [PubMed: 11162410]
- Vozza A, Blanco E, Palmieri L, Palmieri F. Identification of the mitochondrial GTP/GDP transporter in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 2004; 279:20850–20857. [PubMed: 14998997]
- Wiedemann N, Urzica E, Guiard B, Müller H, Lohaus C, Meyer HE, Ryan MT, Meisinger C, Mühlhoff U, Lill R, Pfanner N. Essential role of Isd11 in mitochondrial iron-sulfur cluster synthesis on Isu scaffold proteins. *EMBO J.* 2006; 25:184–195. [PubMed: 16341089]
- Yoon H, Zhang Y, Pain J, Lyver ER, Lesuisse E, Pain D, Dancis A. Rim2, a pyrimidine nucleotide exchanger, is needed for iron utilization in mitochondria. *Biochem. J.* 2011; 440:137–146. [PubMed: 21777202]
- Zheng L, White RH, Cash VL, Dean DR. Mechanism for the desulfurization of L-cysteine catalyzed by the nifS gene product. *Biochemistry.* 1994; 33:4714–4720. [PubMed: 8161529]
- Zheng L, White RH, Cash VL, Jack RF, Dean DR. Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. *Proc. Natl. Acad. Sci. USA.* 1993; 90:2754–2758. [PubMed: 8464885]

Highlights

- Fe-S cluster biogenesis in mitochondria is essential for cell survival.
- The process requires sulfur supply from cysteine by the enzyme cysteine desulfurase.
- A genuine enzyme-bound persulfide intermediate *en route* to Fe-S cluster synthesis has been identified in isolated mitochondria.

Fig. 1.

Persulfide formation by the purified Nfs1p•Isd11p complex. (A) Nfs1p-His₆ together with Isd11p (Nfs1p•Isd11p) or *A. vinelandii* NifS-His₆ (Av NifS) was expressed in bacteria and purified by Ni-NTA chromatography. Samples were treated with SDS-loading buffer containing DTT (10 mM) and analyzed by SDS-PAGE followed by Coomassie Blue staining. The molecular mass of prestained protein standards (Std) is indicated in kDa. (B) The Nfs1p•Isd11p complex (50 ng) or Av NifS (25 ng) was incubated with PLP (0.15 mM), NaCl (0.15 M) and [³⁵S]cysteine (10 μCi) for 15 min at 30°C, followed by precipitation with 10% TCA. Samples were solubilized with SDS loading buffer with or without DTT (10 mM) as indicated, and analyzed by SDS-PAGE followed by autoradiography. Data are from the same autoradiograph, and the dividing line indicates removal of empty lanes in between samples, without or with DTT. (C) Increasing protein amounts of the Nfs1p•Isd11p complex were incubated with PLP, NaCl and [³⁵S]cysteine, and samples were analyzed under non-reducing conditions as in (B) above.

Fig. 2. Detection of Nfs1p by immunoblotting under non-reducing conditions. The Nfs1p•Isd11p complex (50 ng) was incubated for 10 min on ice in HS buffer, with or without salt (0.15 M NaCl). Proteins were precipitated with TCA and the pellets were solubilized with SDS loading buffer, with or without DTT as indicated. One tenth of the samples were analyzed by SDS-PAGE followed by immunoblotting with anti-Nfs1p (Panel 1) and anti-Isd11p (Panel 2) antibodies, respectively. Likewise, wild-type (WT) mitochondria were incubated for 10 min on ice in HS buffer, with or without salt (0.15 M NaCl). Following centrifugation at $15,000 \times g$ for 5 min at $4^{\circ}C$, the mitochondrial pellets were solubilized with SDS loading buffer, with or without DTT as indicated. Samples were analyzed by SDS-PAGE and immunoblotting using anti-Nfs1p (Panel 3) and anti-Isd11p (Panel 4), respectively.

Fig. 3.

Nfs1p-bound persulfide in isolated mitochondria. (A) Nucleotide-depleted mitochondria (200 μ g of proteins) were incubated at 30°C for 15 min in HS buffer containing 10 mM Mg(OAc)₂, 40 mM KOAc, and 10 μ Ci [³⁵S]cysteine in a total volume of 50 μ l. Samples were diluted with HS buffer containing 0.15 M NaCl, left on ice for 10 min, and then centrifuged at 15,000 $\times g$ for 10 min at 4°C to obtain the mitochondrial pellets. The purified Nfs1p•Isd11p complex (50 ng) was used as a positive control (lane 1). As indicated, samples were solubilized with SDS loading buffer with or without DTT (10 mM), and analyzed by SDS-PAGE followed by autoradiography (top panel). Nfs1p-depleted mitochondria are indicated by Nfs1p with a downward arrowhead. The Nfs1p•Isd11p complex (5 ng) and identical mitochondrial samples as above were also analyzed by immunoblotting using anti-Nfs1p (middle panel) and anti-Isd11p (bottom panel) antibodies. Data are from the same autoradiograph (top panel) or blots (middle and bottom panels). The dividing line indicates removal of other samples and/or empty lanes in between. (B) Nucleotide-depleted mitochondria were incubated on ice for 10 min with or without chloramphenicol (0.1 mg/ml). Samples were supplemented with [³⁵S]cysteine and assayed for persulfide formation by non-reducing SDS-PAGE and autoradiography (top panel) as in (A) above. Identical samples were also analyzed for Nfs1p (middle panel) and Isd11p (bottom panel) protein levels by immunoblotting. (C) Reactions were performed in duplicate. Nucleotide-depleted mitochondria were supplemented with [³⁵S]cysteine, diluted with buffer containing salt,

incubated on ice, and then centrifuged to recover mitochondria as in (A) above. One set of reactions was analyzed with no further manipulation (“Total”), another after immunoprecipitation with anti-Nfs1p antibodies (IP). All samples were analyzed by the same non-reducing SDS gel followed by autoradiography and data are from the same autoradiograph. The dividing line indicates removal of other samples and/or empty lanes in between.

Fig. 4. Characterization of the persulfide formed on Nfs1p in isolated mitochondria. (A) Nucleotide-depleted mitochondria were mixed with 10 μ Ci [³⁵S]cysteine in HS buffer containing 10 mM Mg(OAc)₂ and 40 mM KOAc in a final volume of 50 μ l. Following incubation at 30°C for 5-30 min, samples were diluted with HS buffer containing 0.15 M NaCl, left on ice for 10 min, and then centrifuged at 15,000 $\times g$ for 10 min at 4°C. The mitochondrial pellets thus obtained were analyzed by non-reducing SDS-PAGE followed by autoradiography. The intensity of Nfs1p-S-³⁵SH in wild-type (WT) mitochondria at 30-min time point (lane 4) was considered 100%. Identical samples were also analyzed by

immunoblotting using anti-Nfs1p antibodies (bottom panel). (B) Mitochondria in HS buffer (50 μ l) were incubated with or without increasing concentrations of NEM for 10 min at 30°C. Following addition of DTT (10 mM), incubation at 30°C was continued for another 5 min to neutralize free NEM. Samples were then diluted with HS buffer and centrifuged at 15,000 $\times g$ for 5 min at 4°C. The mitochondrial pellets thus obtained were resuspended in HS buffer, incubated with 10 μ Ci [35 S]cysteine for 15 min at 30°C, and assayed for persulfide formation as in (A) above. The intensity of Nfs1p-S- 35 SH in NEM-untreated WT mitochondria (lane 1) was considered 100% (top panel). The bottom panel shows an immunoblot of identical samples for the Nfs1p protein. (C) *A. vinelandii* NifS (25 ng) was incubated with increasing concentrations of NEM for 10 min at 30°C in a final volume of 50 μ l. After addition of DTT (10 mM) incubation at 30°C was continued for another 5 min. After addition of saturated ammonium sulfate to a final concentration of 67%, samples were incubated for 1 h on ice, and centrifuged at 15,000 $\times g$ for 30 min. The protein precipitates were solubilized with buffer A and incubated with PLP (0.15 mM), NaCl (0.15 M) and [35 S]cysteine (10 μ Ci) for 15 min at 30°C. Following TCA precipitation, samples were analyzed by non-reducing SDS-PAGE and autoradiography. The intensity of NifS-S- 35 SH for NEM-untreated sample (lane 1) was considered 100%. (D) The purified Nfs1p•Isd11p complex (50 ng) was treated with increasing concentrations of NEM as indicated and samples were processed as in (C) above. The intensity of Nfs1p-S- 35 SH for NEM-untreated Nfs1p•Isd11p (lane 1) was considered 100% (top panel). One tenth of each sample was analyzed by immunoblotting using anti-Nfs1p antibodies (bottom panel).

Fig. 5. Nucleotide-dependent use of the Nfs1p persulfide for Fe-S cluster formation in isolated mitochondria. (A) Nucleotide-depleted mitochondria were incubated at 30°C for 30 min with 10 μ Ci [³⁵S]cysteine in HS buffer containing 10 mM Mg(OAc)₂ and 40 mM KOAc. A mixture of nucleotides (4 mM ATP, 1 mM GTP, 2 mM NADH) and ferrous ascorbate (Fe²⁺; 10 μ M) was included as indicated. Left panel: Samples were analyzed for persulfide levels by non-reducing SDS-PAGE and autoradiography as in Fig. 3A. The intensity of Nfs1p-S-³⁵S in WT mitochondria with no added nucleotides or iron (lane 1) was considered 100%. Identical samples were also analyzed by immunoblotting using anti-Nfs1p antibodies. Right panel: Samples were analyzed for radiolabeling of aconitase (Aco1p) by native PAGE

and autoradiography as described in Materials and methods (section 2.4). The intensity of Aco1p [Fe-³⁵S] in WT mitochondria in the presence of added nucleotides and iron (lane 2) was considered 100%. Identical samples were also analyzed by immunoblotting using anti-Aco1p antibodies. (B) Wild-type mitochondria were incubated with or without NEM (1 mM) for 10 min at 30°C. After addition of DTT (10 mM), incubation at 30°C was continued for another 5 min. Samples were then diluted with HS buffer and centrifuged at 15,000 × *g* for 5 min at 4°C. The mitochondrial pellets thus obtained were resuspended in HS buffer, and supplemented with nucleotides (4 mM ATP, 1 mM GTP, 2 mM NADH) and ferrous ascorbate (Fe²⁺; 10 μM) as indicated. Following addition of 10 μCi [³⁵S]cysteine, reaction mixtures were incubated for 30 min at 30°C. Samples were analyzed for Nfs1p persulfide formation (top panel) and Nfs1p protein levels (bottom panel). The intensity of Nfs1p-S-³⁵SH in NEM-untreated WT mitochondria in the absence of added nucleotides and iron (top panel, lane 1) was considered 100%. (C) An identical experiment was performed as in (B), and samples were analyzed for Fe-S cluster loading of aconitase (top panel), and Aco1p protein levels by immunoblotting (bottom panel). The intensity of Aco1p [Fe-³⁵S] in NEM - untreated WT mitochondria in the presence of added nucleotides and iron (top panel, lane 2) was considered 100%.

Fig. 6.

Iron-dependent use of the Nfs1p persulfide for Fe-S cluster formation in isolated mitochondria. Nucleotide-depleted mitochondria in HS buffer were incubated with or without 2 mM *o*-phenanthroline for 10 min on ice (1st step). Samples were diluted with HS buffer and centrifuged at 15,000 × *g* for 5 min at 4°C. The mitochondrial pellets thus obtained were resuspended in HS buffer, and supplemented with nucleotides (4 mM ATP, 1 mM GTP, 2 mM NADH) and/or ferrous ascorbate (Fe²⁺; 10 μM) as indicated (2nd step). After addition of 10 μCi [³⁵S]cysteine, reaction mixtures were incubated for 30 min at 30°C. Samples were analyzed for persulfide formation and Nfs1p protein levels (left panel), and Fe-S cluster loading of aconitase and Aco1p protein levels (right panel). The intensity of Nfs1p-S-³⁵SH in *o*-phenanthroline-untreated WT mitochondria in the absence of added nucleotides or iron (left panel, lane 1) was considered 100%. The intensity of Aco1p [Fe-³⁵S] in *o*-phenanthroline-untreated WT mitochondria in the presence of added nucleotides and iron (right panel, lane 3) was considered 100%.