Isd11p Protein Activates the Mitochondrial Cysteine Desulfurase Nfs1p Protein*

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Background: In yeast, Nfs1p is the only known cysteine desulfurase in mitochondria.
Results: Nfs1p is completely inactive on its own and requires an accessory protein, Isd11p, for activity.
Conclusion: Binding of Isd11p may induce a conformational change in Nfs1p, thereby activating the enzyme.
Significance: Isd11p-mediated activation of Nfs1p is critical for vital cellular and mitochondrial functions.

Cysteine desulfurases perform pyridoxal phosphate (PLP)-dependent desulfuration of cysteine. The key steps of the enzymatic cycle include substrate binding to PLP, formation of a covalent persulfide intermediate at the active site cysteine, and transfer of sulfur to recipients for use in various metabolic pathways. In Saccharomyces cerevisiae, the cysteine desulfurase Nfs1p and an accessory protein, Isd11p, are found primarily in mitochondria, and both are essential for cell viability. Although cysteine desulfurases are conserved from bacteria to humans, Isd11p is found only in eukaryotes and not in prokaryotes. Here we show that Isd11p activates Nfs1p. The enzyme without Isd11p is found only in eukaryotes, and thus separating these two functions of Isd11p. A mutant of Isd11p changed to 15AAA17 was able to bind but failed to activate Nfs1p, able to abrogate sulfur delivery for Fe-S cluster synthesis without affecting the corresponding process for thiolation of some tRNAs, demonstrating the specificity of this transfer step (8). Recent studies have pointed to a second portion of the prokaryotic cysteine desulfurase reaction involving direct sulfur transfer from the persulfide at the active site to specific recipients. Mutations in the peptide loop containing the catalytic site are able to abrogate sulfur delivery for Fe-S cluster synthesis without affecting the corresponding process for thiolation of some tRNAs, demonstrating the specificity of this transfer step (8).

The structures of Escherichia coli IscS (9) and Thermotoga maritima NifS (10) are similar. They form homodimers and

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§ The abbreviations used are: PLP, pyridoxal phosphate; NEM, N-ethylmaleimide; Ni-NTA, nickel-nitrilotriacetic acid.

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belong to the α-family of PLP-dependent enzymes. Each monomer contains two domains: the larger harboring the PLP cofactor and the smaller containing the active site cysteine in the middle of a highly flexible loop. The PLP cofactor is situated at the base of a pocket near the surface of the protein, and the pocket forms the substrate-binding site. The loop with the active site cysteine is not well resolved in these structures. However, in the IscS crystals with partially ordered loop, the active site cysteine appears >17 Å away from the PLP cofactor. This implies that large conformational changes in this region would be needed during catalysis (9). To form the persulfide, the active site cysteine would have to rotate toward the PLP site, whereas delivery of the persulfide might involve movement away from the substrate-binding site. No structures of eukaryotic cysteine desulfurases have been solved to date.

In eukaryotic cells such as the yeast *Saccharomyces cerevisiae*, the cysteine desulfurase is encoded by a single gene, *NFS1*. The Nfs1p protein is found primarily in mitochondria (11), although a small amount appears to function in extramitochondrial locations (12). Nfs1p is essential for cell viability. It is required for activities of Fe-S cluster proteins (11, 13). Point mutations in the conserved lysine for PLP attachment or in the conserved active site cysteine result in cell inviability, suggesting that Nfs1p-mediated persulfide formation is critical for Fe-S cluster synthesis (11). This notion is consistent with the observation that a hypomorphic *nfs1* mutant exhibits nuclear iron uptake and accumulates iron in mitochondria, similar to those observed in other yeast mutants deficient in mitochondrial Fe-S cluster assembly (20), no prokaryotic homolog has been found. This is remarkable because other components of this machinery have homologs in bacteria. Isd11p may, therefore, perform a unique function in mitochondria that is not needed in bacteria. Isd11p has been found to associate with Nfs1p in mitochondria, forming a complex (Nfs1p-Isd11p) of approximately 200 kDa. A temperature-sensitive allele or Isd11p-depleted cells show deficiencies in Fe-S cluster protein activities (18, 19). Likewise, knockdown of the corresponding Isd11p homologs in *Trypanosoma brucei* (21) and *HeLa* (22) cells results in inactivation of Fe-S cluster-containing proteins. However, the function of Isd11p has not been determined, and consequently, the reason for Fe-S cluster deficiency in Isd11p-depleted mitochondria remains elusive. Iron-sulfur cluster assembly in mitochondria is a highly complex process requiring multiple proteins (23–25). Here we used purified proteins to directly determine the role of Isd11p in Nfs1p activity. We demonstrate that Isd11p is required for the Nfs1p cysteine desulfurase activity. Binding of Isd11p likely induces a conformational change in the enzyme that allows persulfide formation at the active site cysteine.

**EXPERIMENTAL PROCEDURES**

**Expression of Proteins in Bacteria**—The ORF for the mature form of Nfs1p (lacking the amino-terminal 36 amino acids of the corresponding precursor protein) was amplified by PCR from a yeast genomic library using appropriate primers so that the resulting product was NdeI-ORF-XhoI. The *ISD11* ORF was also amplified in a similar manner. The PCR products were digested with NdeI and XhoI, cloned into the same sites of pET21b (Novagen), and sequenced. This introduces a His$_6$ tag in frame at the C terminus of the proteins. The plasmid pT7–7/NifS (pDB551) for expression of *A. vinelandii* NifS was a gift from Dr. Dennis R. Dean (4). The ORF was modified by introduction of a 5′ Ndel restriction site and a 3′ His$_6$ tag followed by a stop codon and BamHI site. The resulting plasmid was called pT7–7/NifS–His$_6$–stop–BamHI.

The plasmid pST39 (a gift from Dr. Song J. Song) corresponding to Ndel-mature Nfs1p-His$_6$ was digested into pET3aTr using NdeI–BamHI. The NdeI–BamHI fragment containing the 5′ Shine-Dalgarino codon and surrounding regions was moved to the pT7–7/NifS–His$_6$–stop–BamHI fragment of cells lacking the Cys desulfurase. The HindIII fragment of the resulting plasmid was cloned into the corresponding sites of pET21b/Isd11p-His$_6$ and also in plasmid 20–38 containing both Nfs1p-His$_6$ and Isd11p. The mutations were confirmed by sequencing.

All proteins used for our studies described here were expressed in BL21 (DE3) Codon Plus cells (Stratagene). These cells, carrying different plasmids, were initially cultured in Lysogeny Broth supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol to A$_{600}$ of approximately 0.6. Conditions for expression of proteins were optimized so that the majority of each protein was in soluble form (27, 28). Variations included temperature and period of induction and also the concentration of the inducer isopropyl-1-thio-β-D-galactopyranoside. Expression of Nfs1p-His$_6$ alone or together with Isd11p (with or without mutation in the $^{15}$LYK$^{17}$ motif) was carried out in the presence of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 24 h at 20 °C. Isd11p-His$_6$ by itself (with or without mutation) was expressed with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 72 h at 25 °C, and in this case, 0.5 mM sorbitol was included during induction to further enhance the solubility of the expressed protein.
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The mitochondria from A. vinelandii NifS-His, was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. For Nfs1p-His, alone, Nfs1p-His, together with Isd11p (Nfs1p-Isd11p), and A. vinelandii Nifs, cells were harvested and washed with buffer A (50 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 10% glycerol, 1 mM PMSF). Cells in buffer A were treated with lysozyme (50 µg/ml) for 30 min on ice and then disrupted using a probe sonicator (Branson Sonifier 450) by six continuous 15 s bursts at 2 min intervals while keeping the samples on ice. Subsequent steps were carried out at 4 °C. Cell lysates were centrifuged at 12,000 × g for 30 min, and the supernatant fraction was incubated with Ni NTA agarose by end-over-end mixing for 3 h. After washing the resin with buffer A containing 10 mM imidazole, bound proteins were eluted with 0.4 M imidazole for 3 h. After washing the resin with buffer A containing 10 mM imidazole, bound proteins were eluted with 0.4 M imidazole in buffer A. Purified proteins were stored in aliquots at −80 °C until further use. Isd11p-His, was also purified and stored exactly the same way, but NaCl was left out of buffer A.

Sucrose Density Gradient Centrifugation—Mitochondria were purified from a wild-type S. cerevisiae strain D273-10B (ATCC 24657) (29). Mitochondria were suspended in buffer B (50 mM Tris/HCl (pH 7.5), 2 mM MgCl2, 2 mM DTT) containing 1 mM ATP and 1 mM NADH, and membranes were ruptured by mild sonication. Following addition of KCl (0.25 M final) to release membrane-associated Nfs1p and Isd11p, samples were centrifuged at 18,000 × g for 15 min. The supernatant was loaded on a 5–16% sucrose gradient in buffer B containing 0.25 M KCl and centrifuged at 4 °C for 22 h at 285,000 × g in SW40 Ti rotor). Twenty fractions (approximately 0.62 ml each) were collected from the top of the centrifuge tube. Alternate fractions were analyzed by SDS-PAGE followed by immunoblotting using antibodies against Nfs1p and Isd11p because of its association with the PLP cofactor. The purified complex was yellow in color, and the absorbance of the supernatant was measured at 400 nm.

Cysteine Desulfurase Assays—A typical reaction mixture (50 µl) contained proteins in buffer C (20 mM Hepes/KOH (pH 7.5), 0.6 M sorbitol, 0.15 M NaCl) containing 150 µCi [35S]cysteine (0.1 Ci/mM) for 30 min on ice and then disrupted by six continuous 15 s bursts of mild sonication. Following addition of KCl (0.25 M final) to release membrane-associated Nfs1p and Isd11p, samples were centrifuged for 12,000 × g for 30 min, and the supernatant fraction was incubated for 1 h at 37 °C. The reaction was stopped by addition of 6% NaOH (50 µl) and water (250 µl). Sulfide released was measured as described (31). Briefly, 125 µl of 0.1% DPD (N,N-dimethyI-p-phenylenediamine dihydrochloride in 5N HCl) and 50 µl of 11.5 mM FeCl3 were added, followed by incubation for 20 min at 37 °C. Samples were centrifuged at 15,000 × g for 5 min, and the absorbance of the supernatant was measured at 670 nm.

RESULTS

Bacterial Expression of Proteins and Characterization of the Nfs1p-Isd11p Complex—The precursor form of the yeast mitochondrial Nfs1p contains 497 amino acids, including the 36-amino acid-long targeting signal at the N terminus of the protein. Upon import into mitochondria, the targeting signal is proteolytically processed in two steps. The first 33 amino acids are removed by a single PEPTIDE PROTEASE (30), while the second step is performed by an as yet unidentified peptidease, Icp55p (32). At the time of previous studies involving bacterial expression of Nfs1p (33), the cleavable targeting signal was arbitrarily chosen as residues 61–93 (34). For our studies, the mature form of Nfs1p, consisting of the 36 amino acids of the targeting signal, was expressed and purified from E. coli. The protein was purified to homogeneity by Ni-NTA affinity chromatography (Fig. 1A). The proteins Nfs1p-His, and A. vinelandii Nifs, were separately expressed in E. coli. Nfs1p was solubilized with buffer A and assayed. These variations are outlined in the corresponding figures. Radiolabeled protein bands were quantitated using the Adobe Photoshop CS4 software.

The molecular nature and mass of the bacterially expressed Nfs1p-Isd11p complex were then compared with those of the mitochondrial Nfs1p-Isd11p complex by sucrose density gradient centrifugation. Fractions were analyzed by SDS-PAGE followed by immunoblotting using antibodies against Nfs1p and Isd11p. Mitochondrial Nfs1p and Isd11p were found to form a peak in fractions 9–11 (Fig. 1B, panels 1 and 2), and neither Nfs1p nor Isd11p was detected elsewhere in the gradient. On the basis of the migration of standard proteins, the molecular mass of the mitochondrial complex was calculated to be approximately 200 kDa. These results are in good agreement with FPLC or Blue Native gel analysis of the mitochondrial complex (18, 19). Interestingly, bacterially expressed and purified Nfs1p-Isd11p complex also formed a major peak in fractions 9–11 (Fig. 1B, panels 3 and 4). An additional minor peak was observed in fractions 16–19.
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Isd11p Complex but Not Nfs1p by Itself Exhibits Sulphur Depletion and Persulfide Formation

To assess persulfide formation, purified Nfs1p by itself or together with Isd11p (Nfs1p-Isd11p) was expressed in bacteria and purified by Ni-NTA chromatography. Samples were analyzed by SDS-PAGE under reducing conditions followed by Coomassie Blue staining. The difference in migration of Isd11p in panels 2 and 4 is due to the presence of the His6 tag, respectively. The molecular mass of the protein standards (Std) is indicated in kDa. B, mitochondrial extracts (panels 1 and 2) and the bacterially expressed and purified Nfs1p-Isd11p complex (panels 3 and 4) were examined by sucrose density gradient centrifugation. Alternate fractions were analyzed by SDS-PAGE under reducing conditions, followed by immunoblotting with anti-Nfs1p (panels 1 and 3) and anti-Isd11p (panels 2 and 4) antibodies. The molecular mass of the protein standards used for the sucrose gradient centrifugation were carbonic anhydrase (3.15), bovine serum albumin (4.4S), β-amylose (9.5S) and catalase (11.3S). Yellow arrowheads indicate peak fractions for standards as judged by SDS-PAGE followed by Coomassie Blue staining.

FIGURE 1. Analysis of proteins expressed in bacteria. A, Nfs1p-His6, by itself or together with Isd11p (Nfs1p-Isd11p), A. vinelandii (Av) NifS-His6, and Isd11p-His6 were expressed in bacteria and purified by Ni-NTA chromatography. The difference in migration of Isd11p in lanes 2 and 4 is due to the presence of the His6 tag, respectively. The molecular mass of the protein standards (Std) is indicated in kDa. B, mitochondrial extracts (panels 1 and 2) and the bacterially expressed and purified Nfs1p-Isd11p complex (panels 3 and 4) were examined by sucrose density gradient centrifugation. Alternate fractions were analyzed by SDS-PAGE under reducing conditions, followed by immunoblotting with anti-Nfs1p (panels 1 and 3) and anti-Isd11p (panels 2 and 4) antibodies. The molecular mass of the protein standards used for the sucrose gradient centrifugation were carbonic anhydrase (3.15), bovine serum albumin (4.4S), β-amylose (9.5S) and catalase (11.3S). Yellow arrowheads indicate peak fractions for standards as judged by SDS-PAGE followed by Coomassie Blue staining.
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or presence of added Isd11p and analyzed by non-reducing SDS-PAGE followed by autoradiography. Although Nfs1p was not radiolabeled in the absence of Isd11p (Fig. 3A, lane 2), addition of Isd11p resulted in strong radiolabeling (Fig. 3A, lane 3). This radiolabeling was due to $^{35}$S persulfide formation on the enzyme because the Nfs1p-associated $^{35}$S signal was completely lost upon treatment with DTT (Fig. 3A, lane 5). Interestingly, the efficiency of $^{35}$S persulfide formation on Nfs1p with Isd11p added separately (Fig. 3A, lane 3) was comparable with that of bacterially coexpressed and preformed Nfs1p/Isd11p complex (lane 1). As expected, no radiolabeled band was detected in samples containing only Isd11p (Fig. 3A, lane 4).

FIGURE 2. The Nfs1p/Isd11p complex, but not Nfs1p alone, shows enzymatic activity. A, different amounts of the Nfs1p/Isd11p complex or Nfs1p alone were incubated with PLP (150 μM) and $^{[35]S}$cysteine for 10 min at 30 °C. Proteins were precipitated with TCA, and samples were solubilized with SDS loading buffer containing no reducing agent (lanes 1–4). DTT (10 mM) was added to the SDS sample buffer only as indicated (lane 5). Samples were analyzed by SDS-PAGE followed by autoradiography. B, the Nfs1p/Isd11p complex (100 ng) was incubated with $^{[35]S}$cysteine (5 μCi) in the presence of increasing concentrations of PLP, and samples were analyzed by non-reducing SDS-PAGE and autoradiography as in A. The intensity of Nfs1p-S-$^{35}$SH in the presence of 500 μM PLP (lane 5) was considered 100%. C, the Nfs1p/Isd11p complex (100 ng) was incubated with increasing concentrations of $^{[35]S}$cysteine in the presence of a fixed concentration of PLP (150 μM), and samples were analyzed by non-reducing SDS-PAGE and autoradiography as in A. The intensity of Nfs1p-S-$^{35}$SH in the presence of 1 μM $^{[35]S}$cysteine (lane 4) was considered 100%. D, different amounts of the Nfs1p/Isd11p complex were incubated with PLP (150 μM) and $^{[35]S}$cysteine (5 μCi) for 10 min either on ice or at 25 °C, and samples were analyzed. The intensity of Nfs1p-S-$^{35}$SH observed with 250 ng of the complex at 25 °C (lane 8) was considered 100%. E, the Nfs1p/Isd11p complex (100 ng) was incubated with PLP (150 μM) and $^{[35]S}$cysteine (5 μCi) on ice for different periods of time, and samples were analyzed. The intensity of Nfs1p-S-$^{35}$SH at 600 s time point (lane 5) was considered 100%. F, various amounts of the Nfs1p/Isd11p complex or Nfs1p alone were assessed for cysteine desulfurase activity using a colorimetric assay for sulfide release in the presence of L-cysteine, DTT, and PLP as described under “Experimental Procedures.”
These results suggest that individually purified Nfs1p and Isd11p readily interact with each other just as they do when coexpressed in bacteria and that such an interaction activates the enzyme for cysteine desulfurase activity. The activation of Nfs1p was enhanced with increasing amounts of Isd11p added (Fig. 3B), and the persulfide formation was both PLP- (C) and temperature-dependent (D), just like in the case of the preformed and purified Nfs1p/Isd11p complex.

Isd11p-independent and Isd11p-dependent Steps in Persulfide Formation on Nfs1p—We sought to determine how Isd11p turns on Nfs1p activity. For brevity, the cysteine desulfurase activity of Nfs1p can be divided into two stages: binding of the substrate cysteine to the enzyme for cysteine desulfurase activity (Fig. 3B), and the persulfide formation (Fig. 3C and D), just like with Nfs1p/Isd11p complex.

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A. Nfs1p + PLP + {[35S]cysteine} 1st step 
   Incubated 
   Free PLP and 
   β[35S]cysteine removed 
   2nd step 
   Non-reducing 
   SDS-PAGE 
   PLP added back and samples incubated with or without Isd11p

Nfs1p (1st step) | 100 | 200 | 100 | 200 | 200
[35S]cysteine (1st step) | + | + | + | + | +
Isd11p (2nd step) | - | - | 100 | 200 | 200
Nfs1p-S-[35S]SH | 1 | 2 | 3 | 4 | 5 | 6
% Relative intensity | 0 | 0 | 18 | 48 | 80 | 100

B. Nfs1p (1st step) | + | + | + | + 
PLP (1st step) | + | + | - | -
[35S]cysteine (1st step) | + | + | + | +
Isd11p (2nd step) | + | + | + | +
PLP (2nd step) | - | - | - | -
Nfs1p-S-[35S]SH | 1 | 2 | 100 | 0
% Relative intensity | 0 | 0 | 18 | 48 | 80 | 100

C. Nfs1p (ng) | 100 | 200 | 100 | 200 | 200
Nfs1p • Isd11p (ng) | + | + | + | + | +
Nfs1p-S-[35S]SH | 1 | 2 | 100 | 0
% Relative intensity | 0 | 0 | 18 | 48 | 80 | 100

formation was detected only when PLP was included in the first step, regardless of PLP addition during the second step. These results confirm that Nfs1p by itself can bind cysteine in the absence of Isd11p but that PLP must be present. The substrate cysteine thus bound to the enzyme can be utilized for persulfide generation in the presence of Isd11p, and this process can occur with no further addition of PLP. In a different experiment (Fig. 4C), addition of Nfs1p by itself was found to inhibit [35S]persulfide formation by the Nfs1p-Isd11p complex in a dose-dependent manner. Most likely, Nfs1p alone efficiently bound [35S]cysteine, thereby making the substrate unavailable for the complex to form persulfide. These results further substantiate our conclusion that Nfs1p by itself can bind cysteine but cannot form persulfide and that the enzyme must be activated by Isd11p for persulfide generation.

Isd11p Protects the Active Site Cysteine of Nfs1p—Isd11p does not contain any cysteine residues. On the other hand, cysteine desulfurases form persulfide at the active site cysteine residue, and modification of the active site cysteine by an alkylating agent inactivates the enzyme (4). This observation prompted us to determine whether the presence of Isd11p has any effect on the active site cysteine of Nfs1p. For this purpose, we first optimized our assay conditions. As in the well studied A. vinelandii NifS as the model enzyme, our purified bacterial enzyme is highly active, demonstrating about three to four times more activity than NifS (as assessed by [35S]persulfide formation, lanes 1–6; or by spectrophotometric assay for sulfide release (DTT present)). Nfs1p was then incubated with 1 mM NEM treatment using various forms of Nfs1p alone, Isd11p samples. The results were striking. Nfs1p by itself was almost completely inhibited at 0.1 mM (Fig. 5A, lane 4). During the second step of the assay, reaction mixtures containing NEM-treated or NEM-untreated Nfs1p alone were supplemented with Isd11p but not the corresponding Nfs1p-Isd11p samples. The results were striking. Nfs1p by itself was much more sensitive to NEM treatment than the Nfs1p-Isd11p complex. For example, Nfs1p treated with 1 mM NEM followed by Isd11p addition failed to generate any detectable persulfide (Fig. 5B, lane 10). In contrast, the preformed Nfs1p-Isd11p complex was only slightly affected by 1 mM NEM treatment (Fig. 5B, lane 4). These results suggest that Isd11p activates Nfs1p in a way that protects the active site cysteine from being modified by NEM. A conformational change in the enzyme would be in agreement with this notion.

An Isd11p Mutant Binds to Nfs1p but Fails to Activate the Enzymatic Activity and to Protect the Active Site Cysteine—Isd11p belongs to the LYR family of proteins that contain the conserved tripeptide LYS/K (18, 19). Other members of this family include the B12 and B44 subunits of mitochondrial complex I of the respiratory chain of various organisms. However, the role of the tripeptide motif in any of these proteins is unknown. We mutated the tripeptide 12LYK17 of Isd11p to 12AAA17, and the results are shown in Table I.
phenotypic defects that occur from this mutation in S. cerevisiae will be published elsewhere.3 The NEM-activated Nfs1p (Isd11p mut) and Nfs1p-His6 were coexpressed in bacteria in [35S]cysteine (5 Ci), incubated at 30 °C for 10 min, and analyzed. The intensity of Nfs1p-S-35SH for NEM-untreated Nfs1p/Isd11p mut versus Nfs1p by itself.

To further validate these notions, we compared NEM sensitivity of the preformed Nfs1p/Isd11p mut complex (100 ng) or Nfs1p by itself (100 ng) was then added only in the Nfs1p/His6 lane 1, lane 2, and lane 3, respectively (Fig. 6A, lane 1, lane 2, and lane 3, respectively). The affinities of Isd11p and the LYK mutant proteins for binding of Isd11p may replace the Isd11p mutant protein in the Nfs1p/His6 complex. Alternatively, the addition of increasing concentrations of NEM to Nfs1p (100 ng) was treated with free NEM for 10 min at 30 °C. Following TCA precipitation, samples were analyzed by non-reducing SDS-PAGE and autoradiography. B, the preformed Nfs1p/Isd11p mut complex was copurified with Nfs1p. The affinities of Isd11p to Nfs1p remaining to be determined. Interestingly, the positions of protein components in the Nfs1p/His6 complex failed to exhibit any cysteine desulfurase activity. By contrast, Nfs1p by itself was completely inactive (Fig. 2, A and F). Higher concentrations of Isd11p may replace the Isd11p mutant protein in the complex, thereby leading to the conformational change in Nfs1p required for its activity.

To further validate these notions, we compared NEM sensitivity of the two complexes: Nfs1p/Isd11p versus Nfs1p/Isd11p mut (Fig. 6C). The latter was much more sensitive than the former. For example, pretreatment with 1 mM NEM mostly inhibited the mutant complex but only slightly the authentic complex. With regard to NEM (1 mM) sensitivity, the Nfs1p/Isd11p mut practically behaved like Nfs1p alone. These results strongly correlate the role of Isd11p in enzyme activation with active site cysteine protection. Mere binding of Isd11p

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amounts (Fig. 3A) and also PLP and temperature dependence (C and D). Third, Nfs1p by itself was able to bind the substrate cysteine in a PLP-dependent manner but failed to form the persulfide (Fig. 4, A and B). In fact, Nfs1p efficiently competed with the Nfs1p/H18528 Isd11p complex for binding to cysteine (Fig. 4C). Remarkably, Nfs1p with prebound cysteine was able to efficiently form the persulfide with the addition of Isd11p (Fig. 4A).

On the basis of these results, we conclude that Isd11p is not required for Nfs1p binding to cysteine (Isd11p-independent step). However, Isd11p is essential for the subsequent step, i.e. formation of the persulfide at the active site cysteine from the bound substrate cysteine (Isd11p-dependent step).

We propose that in the absence of Isd11p, the active site cysteine residue 385 of Nfs1p is not close enough to act on the substrate cysteine bound to PLP at lysine residue 263 and thus fails to form the persulfide. A critical interaction of Isd11p with Nfs1p induces a conformational change in the enzyme that brings the active site cysteine and the substrate cysteine closer, thereby facilitating persulfide formation (Fig. 7).

The notion of Isd11p-mediated conformational change in the enzyme is supported by other independent observations. For example, Isd11p with a mutation in the conserved LYK motif interacted with Nfs1p but failed to activate the enzyme (Fig. 6, A and B). This could be due to the inability of the Isd11p mutant protein to induce the necessary conformational change in the enzyme. Mere binding of the Isd11p mutant was not sufficient to activate the enzyme.

Another supporting evidence for a conformational change in the enzyme is suggested by the differential NEM sensitivity of the active site cysteine. Compared with Nfs1p alone, the Nfs1p/Isd11p complex was more resistant to inactivation by NEM (Fig. 5B). Isd11p may mediate the conformational change in Nfs1p in such a way that the active site cysteine becomes less accessible to modification by NEM. The Isd11p mutant was less effective in protecting the active site cysteine from NEM modification (Fig. 6C), possibly because of its inability to induce the conformational change in the enzyme. From what is known about the bacterial IscS structure (9), the movement of the peptide loop containing the active site cysteine toward the substrate-binding site might very well generate a more compact structure that would better shield the active site from NEM. Another step in the enzymatic cycle of cysteine desulfurases

FIGURE 6. Effects of an Isd11p mutation on interaction with Nfs1p, enzyme activation, and active site cysteine protection. A, the tripeptide15LYK17 of Isd11p was mutated to15AAA17, and the corresponding protein (Isd11p mut) was coexpressed with Nfs1p-His6 in bacteria in a soluble form. The Nfs1p/Isd11p mut complex was purified by Ni-NTA chromatography by SDS-PAGE under reducing conditions followed by autoradiography. The Nfs1p/Isd11p complex (200 ng) without any added Isd11p was analyzed by non-reducing SDS-PAGE followed by autoradiography. B, the Nfs1p/Isd11p complex (200 ng) was incubated with PLP (150 μM) and [35S]cysteine (5 μCi) for 10 min at 30 °C. The Nfs1p/Isd11p mut complex (200 ng) was incubated with PLP (150 μM) and [35S]cysteine (5 μCi) for 10 min at 30 °C. Excess DTT (10 mM) was added to neutralize free NEM, and the protein samples obtained after ammonium sulfate precipitation were solubilized with buffer A. Isd11p (100 ng) was added only to Nfs1p/Isd11p mut samples. All reaction mixtures were subsequently supplemented with PLP (150 μM) and [35S]cysteine (5 μCi) for 10 min at 30 °C. The intensity of Nfs1p-S-35SH for NEM-untreated Nfs1p/Isd11p (lane 1) was considered 100%.
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FIGURE 7. Model for Isd11p-mediated activation of Nfs1p for persulfide formation. A schematic is presented depicting how Isd11p might be involved in Nfs1p activation. The PLP-conjugating lysine 263 and the active site cysteine 385 of Nfs1p correspond to the mature form of the protein in mitochondria (11, 32). See text for details.

Previous studies (18, 19) have overlooked the requirement of Isd11p for Nfs1p activity. Mitochondria isolated from a temperature-sensitive isd11 mutant or Isd11p-depleted cells exhibited Fe-S cluster deficiency. When these mutant mitochondrial extracts were incubated with cysteine and DTT, sulfide production was found to be unaffected or even slightly enhanced. On the basis of these results, it was concluded that Isd11p is not required for the cysteine desulfurase activity of Nfs1p, rather it functions as a stabilizer of Nfs1p. However, no evidence was presented demonstrating that the observed sulfide production in these mutant mitochondria was indeed due to Nfs1p and not due to activities of other proteins/enzymes. In fact, using the same assay, we have seen sulfide production not only in Isd11p-depleted mitochondria but also in Nfs1p-depleted mitochondria. The activity, being present in Nfs1p-depleted mitochondria, cannot be attributed to the Nfs1p cysteine desulfurase. In Isd11p-depleted mitochondria, Nfs1p fails to form [35S]persulfide at its active site. These results are in agreement with the data presented here and will be published elsewhere.

Fe-S cluster biogenesis in mitochondria is an essential and conserved process. It is highly complex, and the mechanistic details remain to be determined. The steps involved must be tightly regulated because both components (iron and sulfur) are toxic when unassembled or present in excess. The Nfs1p/Isd11p complex interacts with the scaffold proteins...
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(Isd11p/Isu2p) for the synthesis of Fe-S cluster intermediates, with the sulfur provided by Nfs1p and the iron donated by the yeast frataxin homolog Yfh1p (23, 25). Recent studies suggest that frataxin interacts with a complex of human Nfs1, Isd11, and Isu2 and serves as an allosteric switch regulating the synthesis of Fe-S clusters (37, 38). The data presented here may point to another level of regulation. For example, Isd11p in mitochondria may reversibly interact with Nfs1p, depending on the demand for Fe-S cluster synthesis, thereby turning the cysteine desulfurase activity on and off. This could be mediated by the availability of mitochondrial cysteine, iron, and/or nucleotides (ATP, GTP, or NADPH) required for Fe-S cluster biogenesis (28, 29, 39). The distribution of Isd11p between the mitochondrial inner membrane and the soluble matrix (18) might also be important for localizing the active cysteine desulfurase to critical locations within mitochondria. Proper regulation of the cysteine desulfurase activity and the role of Isd11p are likely to be relevant to vital cellular functions, including the tricarboxylic acid cycle, respiration, and protein translation.

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