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Functional Characterization of the Eukaryotic Cysteine Desulfurase Nfs1p from *Saccharomyces cerevisiae**[S]

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Previous studies have indicated that the essential protein Nfs1 performs a crucial role in cellular iron-sulfur (Fe/S) protein maturation. The protein is located predominantly in mitochondria, yet low amounts are present in cytosol and nucleus. Here we examined several aspects concerning the molecular function of yeast Nfs1p as a model protein. First, we demonstrated that purified Nfs1p facilitates the *in vitro* assembly of Fe/S proteins by using cysteine as its specific substrate. Thus, eukaryotic Nfs1 is a functional orthologue of the bacterial cysteine desulfurase IscS. Second, we showed that only the mitochondrial version but not the extramitochondrial version of Nfs1p is functional in generating cytosolic and nuclear Fe/S proteins. Mutation of the nuclear targeting signal of Nfs1p did not affect the maturation of cytosolic and nuclear Fe/S proteins, despite a severe growth defect under this condition. Nfs1p could not assemble an Fe/S cluster on the Isu scaffold proteins when they were located in the yeast cytosol. The lack of function of these central Fe/S cluster assembly components suggests that the maturation of extramitochondrial Fe/S protein does not involve functional copies of the mitochondrial Fe/S cluster assembly machinery in the yeast cytosol. Third, the extramitochondrial version of Nfs1p was shown to play a direct role in the thiomodification of tRNAs. Finally, we identified a highly conserved N-terminal β-sheet of Nfs1p as a functionally essential part of the protein. The implication of these findings for the structural stability of Nfs1p and for its targeting mechanism to mitochondria and cytosol/nucleus will be discussed.

NifS-like proteins such as the cysteine desulfurases NifS or IscS of *Azotobacter vinelandii* are ubiquitous, homodimeric proteins that belong to the α-family of pyridoxal 5′-phosphate (PLP)[1]-dependent enzymes (1, 2). They generate elemental sulfur from cysteine via a cysteine persulfide intermediate that is bound to an essential cysteine residue close to the C terminus (3). Sulfur abstraction involves the transient binding of the cysteine substrate to the PLP cofactor via a Schiff base. NifS-like proteins are structured into two domains, the larger bearing the PLP co-factor and the smaller hosting the active site cysteine residue in the middle of a highly flexible loop (4, 5). NifS-like cysteine desulfurases were first identified in nitrogen-fixing bacteria as an essential component of the nitrogen fixation system that is dedicated to the biosynthesis of the iron-sulfur (Fe/S) co-factors of nitrogenase (6). Two other highly conserved systems utilize this class of proteins as sulfur donors for the formation of Fe/S clusters. The iron-sulfur cluster (ISC) assembly system is required for the synthesis of cellular Fe/S proteins in general and is widely distributed throughout the bacterial world (7–9). The sulfur mobilization (SUF) system seems to be specialized for the synthesis and/or repair of cellular Fe/S proteins under oxidative stress and iron-limiting conditions (10–13).

In eukaryotes such as *Saccharomyces cerevisiae*, mitochondria play a central role in the biosynthesis of cellular Fe/S proteins (14, 15). Several components of the mitochondrial ISC assembly machinery including the IscS homologue Nfs1p are related to proteins of the bacterial *isc* operon, indicating the bacterial origin of this important process (16–18). The yeast mitochondrial ISC assembly machinery is essential for the biosynthesis of mitochondrial Fe/S proteins that perform central roles in respiration, amino acid and vitamin biosynthesis, and the citric acid cycle. In addition, it is required for the maturation of cytosolic Fe/S proteins (18–21). The latter process also involves the mitochondrial "ISC export machinery" whose members include the mitochondrial ABC transporter Atm1p, the intermembrane space protein Erv1p, and glutathione (18, 22, 23).

In bacteria and yeast, IscS and Nfs1p form stable complexes with the highly conserved Fe/S assembly proteins IscU and Isu1p, respectively, that were shown to serve as central scaffolds for the *de novo* synthesis of cellular Fe/S clusters by chemical or IscS/NifS-directed reconstitution (9, 24). The cluster can be transferred from IscU to Fe/S apoproteins *in vitro*. In *S. cerevisiae*, the homologous mitochondrial protein pair Isu1p/Isu2p is essential, and loss of function causes severe defects in cellular Fe/S cluster maturation (17, 25, 26). The binding of an Fe/S cluster to Isu1p protein has been demonstrated *in vivo*, and the *de novo* formation of this Fe/S cluster has been shown to require Nfs1p, the yeast adrenodoxin homologue Yah1p, and the yeast frataxin homologue Yfh1p (27). IscU or Isu1p/Isu2p directly interact with the ATP-dependent Hsp70 chaperone Hsc66 (yeast homologue Ssq1p), its cognate J-type co-chaperone Hsc20 (yeast Jac1p), and with Yfh1p, but the biochemical meaning of these associations is still unclear (28–33).

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[S] The on-line version of this article (available at http://www.jbc.org) contains Fig. S1 and Table S1.

† Dr. Gyula Kispal died on March 20, 2003 in a tragic car accident. We all miss him.

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[1] The abbreviations used are: PLP, pyridoxal 5′-phosphate; DTT, dithiothreitol; HA, hemagglutinin-A; ISC, iron-sulfur cluster.

TABLE I
Nfs1p from S. cerevisiae functions as a specific cysteine desulfurase

Deduced molecular mass	47.3 kDa
Native molecular mass[a]	102 ± 5 kDa
Cysteine sulfur transferase activity (cysteine to DTT)	12.7 ± 0.1 nmol s^{2-}/mg/min
Cystine sulfur transferase activity (cystine to DTT)	Not detectable
Cysteine sulfur transferase activity (cysteine to cyanide)	Not detectable
Thiosulfate sulfur transferase activity (thiosulfate to DTT)	Not detectable
Thiosulfate sulfur transferase activity (thiosulfate to cyanide)	Not detectable

[a] Determined by gel filtration.

Bacterial NifS-like cysteine desulfurases serve as versatile sulfur donors in a variety of essential metabolic pathways other than Fe/S protein biosynthesis, including vitamin and tRNA biosynthesis (34–36). IscS of *Escherichia coli* has been shown to display tRNA sulfur transferase activity required for the biosynthesis of 4-thiouridine, the predominant thiolated nucleotide in some tRNAs of bacteria (37, 38). Mutations in proteins involved in Fe/S cluster maturation other than IscS can affect the thiolation pattern of tRNA in bacteria, indicating that for some tRNA species thio-modification crucially involves Fe/S proteins (39, 40).

Nfs1 of eukaryotes is highly homologous to the bacterial NifS-like proteins and is located predominantly in mitochondria (18, 41, 42). In recent years, several lines of evidence have been provided for a multiple cellular localization of this protein. In yeast, genetic evidence has shown that Nfs1p also performs an essential function in the cell nucleus (43). During the preparation of this manuscript, an involvement of Nfs1p in thiouridine modification of tRNA was reported indicating that it mimics the role of IscS-like desulfurases in bacteria (44). In human cells, an extramitochondrial Nfs1 species was detected that was proposed to be required for the maturation of Fe/S proteins in the cytosol (41). The cytosolic form of Nfs1 was smaller than its mitochondrial version, a fact that was explained by alternative start codon utilization of the mRNA transcribed from the single *NFS1* gene in humans.

In this work, we have addressed several functional aspects of Nfs1 in the model organism *S. cerevisiae*. First, we determined the substrate specificity of this protein and compared it with bacterial desulfurases. Second, we investigated the cellular roles of the mitochondrial and extramitochondrial versions of the protein. Finally, we examined the role of the highly conserved N terminus of Nfs1 for its function, and we discuss the implications for the functional integrity of this protein and for its targeting to different cellular compartments.

MATERIALS AND METHODS

Strains and Cell Growth—The following strains of *S. cerevisiae* were used: W303-1A (*MAT***a***, ura3-1, ade2-1, trp1-1, his3-11,15, leu2-3,112*) as wild-type, the promoter exchange mutants Gal-NFS1, (27), Gal-ATM1 (18), and Gal-YAH1 (19). Cells were grown in rich (YP) or minimal (SC) media containing the required carbon sources (45). Yeast cells were transformed with plasmid DNA by the lithium acetate method (46). Complementation analysis in *E. coli* was carried out with strain PK4331 (Δ*iscS*::*kan*^{R}) grown on LB medium supplemented with kanamycin and ampicillin (34).

Fe/S Protein Assembly Mediated by Recombinant Nfs1p—Expression of *S. cerevisiae* Nfs1p in *E. coli* was carried out as described previously (47). *E. coli* strain HMS174 (DE3) harboring plasmid pET15b-NFS1 was grown at 30 °C in LB medium supplemented with 50 μM pyridoxal phosphate and 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested, resuspended in TS buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 75 mM NaCl), and lysed by sonication. The cleared cell extract was fractionated by ammonium sulfate precipitation, and the 35–65% ammonium sulfate fraction was applied to a phenyl-agarose column equilibrated with 15% ammonium sulfate-saturated TS buffer. Nfs1p was eluted by a gradient from 15 to 0% ammonium sulfate and purified further by gel filtration chromatography on a Sephacryl S300 column in 25 mM Tris-HCl, pH 8.0, 50 mM NaCl.

Nfs1p-directed Fe/S protein assembly was carried out by using recombinant ferredoxin from *Thermosynechococcus elongatus* as a reporter (48). The soluble apoprotein was prepared as described (49). For radiolabeling with ^{55}Fe, 10 μg of apoferredoxin was incubated with 10 μg of Nfs1p in buffer A (20 mM Hepes-NaOH, pH 7.4, 0.6 M sorbitol, 150 mM NaCl, 2 mM $MgCl_2$) supplemented with 0.2 mM cysteine, 1 mM sodium ascorbate, 1 mM DTT, 10 μM pyridoxal phosphate, and 5 μCi of $^{55}FeCl_3$ at 25 °C under anaerobic conditions. Reactions were terminated with 2 mM EDTA on ice, and holoferredoxin was recovered by ion-exchange chromatography on Q-Sepharose (48). ^{55}Fe incorporation into ferredoxin was quantified by liquid scintillation counting. For nonradioactive Fe/S cluster assembly, 10 μg of apoferredoxin was incubated for 16 h under anaerobic conditions with 10 μg of Nfs1p in 50 mM Tris-HCl, pH 8, supplemented with 4 mM cysteine, 1 mM sodium ascorbate, 0.3% β-mercaptoethanol, 15 μM pyridoxal phosphate, and 100 μM ferric ammonium citrate. Holoferredoxin was subsequently purified as described above.

^{35}S Incorporation into Cellular tRNA in Vivo—Yeast strains were grown for 16 h in minimal medium supplemented with glucose (SD). Cells (0.5 g) were resuspended in fresh sulfur-free SD medium and were radiolabeled with [^{35}S]methionine/cysteine (50 μCi) for 30 min. Total RNA was extracted by the guanidinium-isothiocyanate method (Qiagen). tRNA fractions were separated from the bulk RNA by denaturing polyacrylamide gel electrophoresis, blotted on nylon membranes, and analyzed by autoradiography. $tRNA^{Lys}$ (UUU) and the mRNA for *ACT1* were detected by Northern blot analysis. The amounts of 25 S and 18 S rRNA were determined by ethidium bromide staining in agarose gels.

Miscellaneous Methods—The following published methods were used: manipulation of DNA and PCR (50); preparation of whole cell lysates by mechanical cell disruption with glass beads and isolation of yeast mitochondria (51); immunostaining and immunoprecipitation (52); *in vivo* labeling of yeast cells with radioactive iron ($^{55}FeCl_3$) and measurement of ^{55}Fe incorporation into Fe/S proteins (18); and enzymatic activities of Nfs1p (53, 54).

RESULTS

Nfs1p Is a Cysteine Desulfurase Capable of Fe/S Protein Formation in Vitro—Nfs1p from *S. cerevisiae* has been shown to be involved in the formation of cellular Fe/S proteins (18). To characterize the enzyme activity of Nfs1p, the protein was overproduced in *E. coli* and purified. Because the exact position of the N-terminal mitochondrial targeting sequence was unknown, residue 68 was arbitrarily chosen as the N terminus of the recombinant protein, a position well before the starting point of significant sequence similarity of NifS-like proteins. Comparison of the electrophoretic mobility of recombinant and mitochondrial Nfs1p indicated that the endogenous mitochondrial protein was slightly larger than the recombinant protein facilitating discrimination between the two species (see below). The purified protein showed the typical absorption spectrum of PLP-containing enzymes (not shown). Gel filtration analysis indicated that the protein behaved as a homodimer (Table I). Both observations are in accordance with results for NifS-like proteins from other species.

In order to characterize the molecular function of Nfs1p, we determined its substrate specificity. Sulfur transferases catalyze the transfer of sulfur atoms to a thiophilic acceptor substrate. Most prominent members are cysteine desulfurases that liberate elemental sulfur from cysteine or cystine, and rhodaneses that catalyze the formation of thiocyanate (rhodanide) by transferring sulfur from thiosulfate to cyanide (1,

55, 56). Purified Nfs1p from *S. cerevisiae* was capable of catalyzing efficient sulfur transfer from cysteine to DTT at a rate similar to that of other NifS-like proteins (Table I). The pH optimum of the reaction was around pH 8.5, in accordance with other desulfurases (not shown). In contrast, only background levels of sulfur transfer from cysteine to cyanide were observed for Nfs1p, and neither thiosulfate nor cystine served as sulfur donors for either cyanide or DTT as an acceptor (Table I). Hence, yeast Nfs1p is a *bona fide* cysteine desulfurase with little or no rhodanese activity. Moreover, the cysteine desulfurase activity of mitochondria isolated from the conditional yeast strain Gal-NFS1 (18) was severely impaired upon depletion of Nfs1p, indicating that Nfs1p is the only mitochondrial enzyme capable of carrying out the enzymatic production of sulfur from cysteine in yeast (not shown).

Bacterial NifS/IscS have been shown to facilitate the formation of Fe/S proteins *in vitro*. In order to verify a similar role for *S. cerevisiae* Nfs1p, a cyanobacterial apoferredoxin was incubated with radioactive ^{55}Fe and cysteine under anaerobic conditions. A time-dependent radiolabeling of apoferredoxin was observed in the presence of recombinant Nfs1p (Fig. 1*A*). The observed iron binding was specific, as only background signals were detected in the absence of cysteine, Nfs1p, or apoferredoxin (Fig. 1*B*). Moreover, the reaction required anaerobic conditions and the presence of a thiol-containing substance such as DTT or β-mercaptoethanol. A similar requirement for thiol-containing compounds was observed previously for the formation of Fe/S proteins in mitochondrial extracts *in vitro* (47). Finally, in order to show that the bound iron was in fact part of an Fe/S cluster, apoferredoxin was incubated under similar conditions with Nfs1p, cysteine, and nonradioactive Fe^{2+}, and the reconstituted protein was purified. The absorption spectrum of the reconstituted ferredoxin was similar to that of the native protein exhibiting typical maxima at 330 and 420 nm which are indicative of a [2Fe-2S] cluster (Fig. 1*C*; 48). Taken together, the data show that recombinant Nfs1p functions as a cysteine desulfurase that is capable of catalyzing Fe/S cluster formation on Fe/S apoproteins *in vitro*.

Nuclear Nfs1p Does Not Contribute to the Maturation of Extramitochondrial Fe/S Proteins—Nfs1p of *S. cerevisiae* and other eukaryotes carry typical N-terminal mitochondrial targeting sequences, and the majority of Nfs1p is located within mitochondria (*cf.* supplemental Fig. S1a). In addition, genetic studies have provided evidence that the protein performs an essential function in the nucleus, although conventional *in situ* localization techniques were unable to detect Nfs1p in the nucleus (43). For nuclear targeting, Nfs1p appears to use a classical nuclear targeting signal in the middle of the protein. Because Nfs1p has been shown to be required for the maturation of both mitochondrial and cytosolic Fe/S proteins (18), it is reasonable to assume that the extramitochondrial Nfs1p may be involved in the maturation of cytosolic and nuclear Fe/S proteins, whereas the mitochondrial protein is required for the maturation of mitochondrial Fe/S proteins. In order to test this possibility, we examined the requirements for mitochondrial *versus* extramitochondrial Nfs1p for Fe/S biogenesis in the cytosol by measuring the *in vivo* incorporation of radioactive ^{55}Fe into the cytosolic Fe/S protein Leu1p. We used the yeast strain Gal-NFS1 that carries the *NFS1* gene under the control of the conditional *GAL1-10* promoter that is induced by galactose and repressed upon growth in the presence of glucose (18). Cells were transformed with plasmids encoding mutant versions of Nfs1p lacking either the mitochondrial or the nuclear targeting signal (supplemental Table S1). Gal-NFS1 cells showed a strong reduction in the amount of ^{55}Fe associated

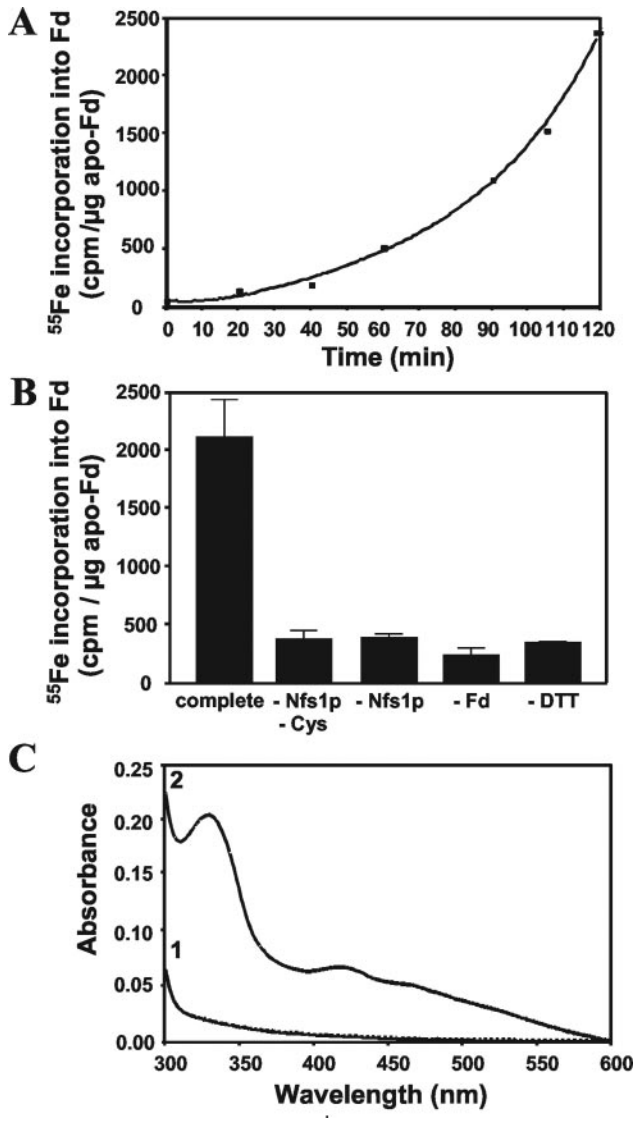

FIG. 1. **Purified Nfs1p from *S. cerevisiae* catalyzes the formation of Fe/S clusters on Fe/S apoproteins.** *A*, apoferredoxin (*Fd*) from *T. elongatus* was incubated with recombinant Nfs1p in the presence of 5 mM cysteine, 1 mM DTT, and ^{55}Fe at 25 °C for the indicated times. Labeling reactions were terminated with EDTA; ferredoxin was reisolated by ion-exchange chromatography, and the incorporation of radioactive ^{55}Fe into ferredoxin was determined by scintillation counting. For each time point, the signal obtained in the absence of Nfs1p was subtracted from the data obtained in the presence of Nfs1p (see *B*). *B*, ^{55}Fe incorporation into apoferredoxin as in *A* omitting the indicated compounds; *C*, UV/visible spectrum of *T. elongatus* apoferredoxin before (*curve 1*) and after (*curve 2*) reconstitution by Nfs1p in the presence of 4 mM cysteine, 0.3% β-mercaptoethanol, and 100 µM Fe^{2+}.

with immunoprecipitated Leu1p upon repression of *NFS1* by growth in the presence of glucose (Fig. 2*A*). As noted previously, Leu1p levels were slightly reduced upon Nfs1p depletion, indicating that the apoform of this protein is highly susceptible to proteolysis (Fig. 2*C*) (18). In accordance with previous results, this defect in the *de novo* maturation of cytosolic Leu1p in Gal-NFS1 cells was not cured by expression of a truncated version of Nfs1p that initiates at Met-85 (18). This species is predominantly located in the cytosol and not in mitochondria, indicating that mitochondrial localization of Nfs1p is essential for the maturation of cytosolic Fe/S proteins (supplemental Fig. S1d). In contrast, Gal-NFS1 cells carrying an additional version of Nfs1p that lacked the nuclear targeting signal (expressed from plasmid pTT-GSR-NFS1-h6; 43), and hence was localized mainly in mitochondria (supplemental Fig. S1f),

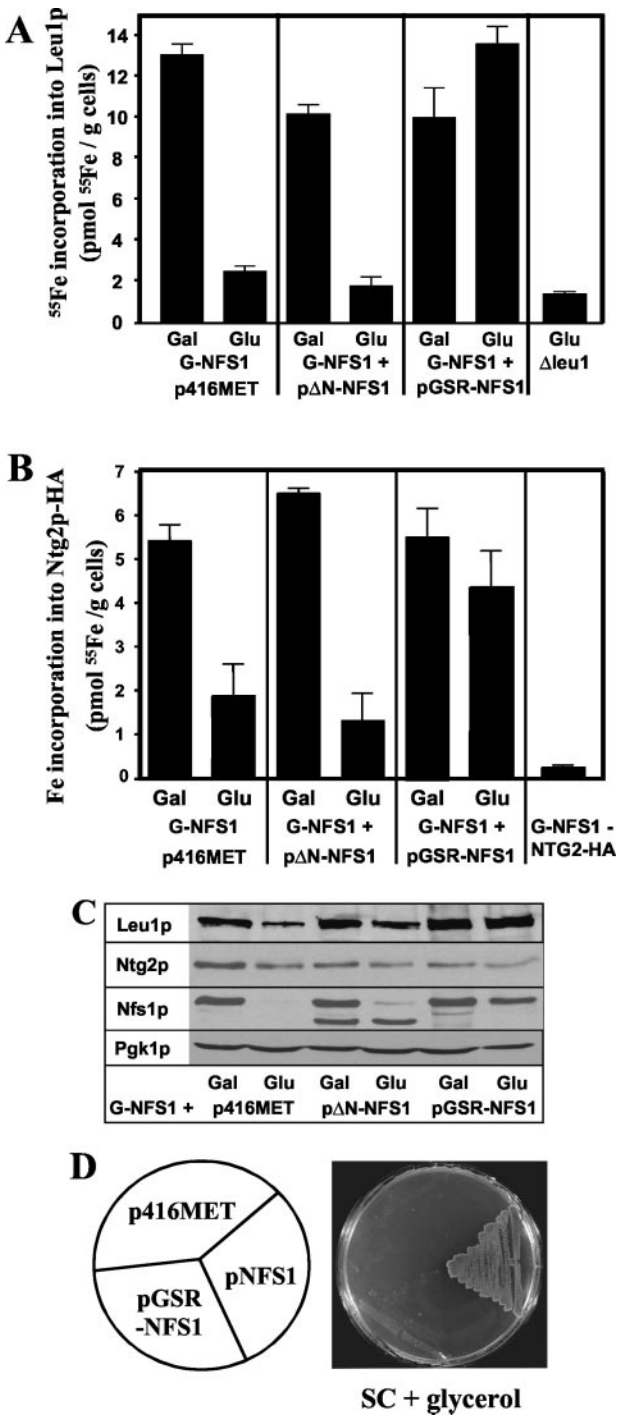

FIG. 2. **The essential nuclear targeting signal of Nfs1p is not required for the maturation of extramitochondrial Fe/S proteins.** Gal-NFS1 cells (*G-NFS1*) lacking (*p416MET*) or expressing a version of Nfs1p that lacks the N-terminal mitochondrial targeting sequence (*pΔN-NFS1*) or a version of Nfs1p that carries a mutated nuclear targeting signal (*pGSR-NFS1*) were grown in iron-poor minimal medium supplemented with either galactose (*Gal*) or glucose (*Glu*) for 16 h. Cells were radiolabeled with ^{55}Fe; cell lysates were prepared, and the *de novo* Fe/S cluster assembly into cytosolic Leu1p (*A*) or the nuclear protein Ntg2p-HA (*B*) was analyzed by immunoprecipitation using specific antibodies (18). Ntg2p-HA contains a hemagglutinin A (HA) tag and was immunoprecipitated with anti-HA antibodies. *Error bars* indicate the standard deviation. Controls for background ^{55}Fe precipitation are shown on the *right* using Δleu1 cells (*A*) or Gal-NFS1 cells (*B*) lacking Ntg2p-HA. *C,* immunostaining of the indicated proteins in cell extracts of Gal-NFS1 cells analyzed in *A* and *B*. Pgk1p is a cytosolic marker protein for the input. *D,* Gal-NFS1 cells harboring plasmids p416MET, pTT-GSR-NFS1, or pNFS1 (see supplemental Table S1) were grown on synthetic minimal medium (SC) supplemented with glycerol at 30 °C.

showed wild-type levels of ^{55}Fe incorporation into Leu1p upon Nfs1p depletion.

By using the same radiolabeling assay, we next investigated the role of extramitochondrial Nfs1p in the maturation of the nuclear DNA repair enzyme Ntg2p. We used a hemagglutinin-A (HA)-tagged version of Ntg2p as reporter that was expressed from plasmid p426GPD. This protein is localized exclusively in the nucleus (57) (supplemental Fig. S1). Similar to Leu1p, the incorporation of ^{55}Fe into Ntg2p-HA in Gal-NFS1 cells declined under *NFS1*-repressing conditions (Fig. 2*B*). This defect was not cured upon expression of the N-terminally truncated, cytosolic/nuclear version of Nfs1p in this strain. Most interestingly, diminished Fe/S cluster assembly on Ntg2-HA did not affect its nuclear localization (supplemental Fig. S1d). Upon expression of the Nfs1p species that lacks the nuclear targeting signal, Gal-NFS1 cells showed almost normal levels of ^{55}Fe incorporation into Ntg2p-HA, even upon depletion of Nfs1p. These data show that nuclear localization of Nfs1p is not required for the maturation of cytosolic and nuclear Fe/S proteins in yeast. However, the introduction of plasmid pTT-GSR-NFS1 into Gal-NFS1 cells did not restore the growth defects of our Gal-NFS1 cells under *NFS1*-repressing conditions (Fig. 2*D*). Hence, the essential character of extramitochondrial Nfs1p cannot be explained by a function in the maturation of extramitochondrial Fe/S proteins.

Human Isu Proteins Do Not Assemble Fe/S Clusters When Expressed in the Yeast Cytosol—Our results clearly demonstrate that mitochondrial localization of Nfs1p was necessary for the maturation of cytosolic and nuclear Fe/S proteins and that nuclear targeting of Nfs1p was not needed for this process. However, it cannot be concluded from these data that GSR-Nfs1p (no nuclear targeting) was quantitatively imported into mitochondria. Minute amounts may remain in the cytosol, where the protein may contribute to the maturation of cytosolic and nuclear Fe/S proteins, in addition to the function of mitochondrial Nfs1p. It seemed technically impossible to us to preclude cytosolic localization of Nfs1p with certainty. In order to circumvent this problem, we investigated whether a potential cytosolic Nfs1p can assemble an Fe/S cluster on the Isu proteins in the yeast cytosol. These proteins serve as indispensable scaffolds for the *de novo* synthesis of Fe/S clusters (24), and Fe/S cluster formation on these proteins can be followed in yeast by radiolabeling experiments *in vivo* (27). We used the human homologues hIsu1 and hIsu2 in these experiments, because the yeast Isu1p protein cannot be mis-targeted to the cytosol (26). In human cells, hIsu2 represents the mitochondrial version of the Isu proteins, whereas small amounts of hIsu1 have been detected in the cytosol (58). Both proteins are thought to be derived from a single gene by differential mRNA splicing. Three yeast expression vectors were constructed (supplemental Table S1) that allowed the synthesis of either a mitochondria-targeted version of hIsu2 as a control (F1β-hIsu2-HA), a cytosolic version of hIsu2 (Δ33-hIsu2-HA), or the cytosolic hIsu1 (hIsu1-HA; 58). For detection and immunoprecipitation, proteins were fused with a C-terminal HA tag. The desired targeting of these proteins to mitochondria or cytosol in yeast has been documented previously (26).

Proteins were synthesized in the conditional yeast strain Gal-ISU1/Δisu2 that allowed the repression of the endogenous yeast *ISU1* by growth in the presence of glucose (26). Similar to the untagged versions, the HA-tagged human Isu proteins restored the growth defect of the Gal-ISU1/Δisu2 strain in the presence of glucose, yet only when targeted to mitochondria (26). In order to investigate whether the human proteins can bind Fe/S clusters in yeast, cells overproducing F1β-hIsu2-HA, Δ33-hIsu2-HA, or hIsu1-HA were radiolabeled with ^{55}Fe, and

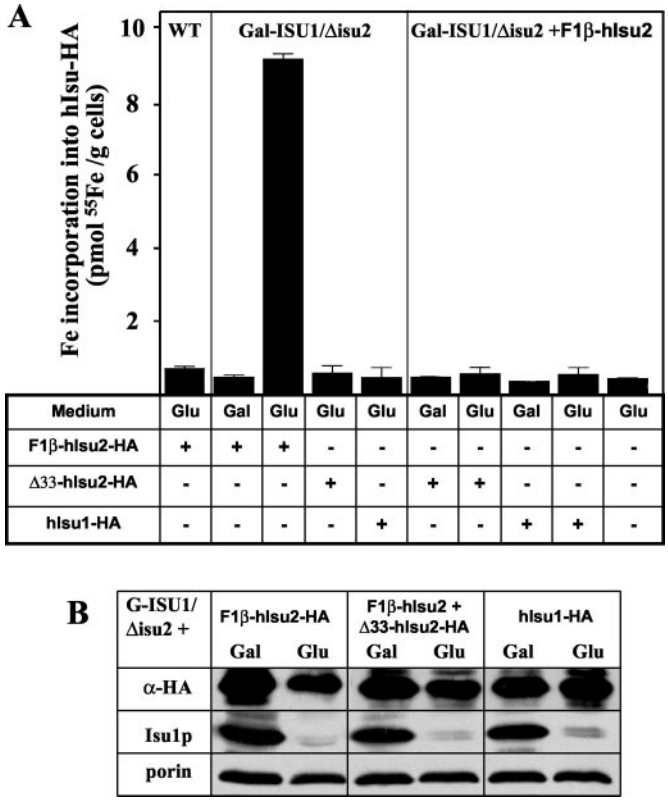

FIG. 3. **The human Isu proteins cannot assemble Fe/S clusters when expressed in the yeast cytosol.** *A*, wild-type (*WT*) and Gal-ISU1/Δisu2 cells overexpressing the indicated human HA-tagged Isu proteins were grown in iron-poor minimal medium supplemented with galactose (*Gal*) or glucose (*Glu*). Cells were radiolabeled with ^{55}Fe for 2 h, and cell lysates were prepared. Human Isu proteins were immunoprecipitated with anti-HA antibodies, and the ^{55}Fe radioactivity associated with the immunobeads was quantified by liquid scintillation counting. *Error bars* indicate the standard deviation. *B*, immunostaining of the indicated proteins in the cellular extracts of Gal-ISU1/Δisu2 cells. Porin is a mitochondrial protein used as an input marker.

the human Isu proteins were immunoprecipitated from cellular extracts using anti-HA antibodies. Little radioactivity was associated with mitochondrial F1β-hIsu2-HA, when the protein was immunoprecipitated from either wild-type or Gal-ISU1/Δisu2 cells cultivated in the presence of galactose (Fig. 3*A, left*), although the protein was strongly expressed in these strains (Fig. 3*B*). However, when Gal-ISU1/Δisu2 cells were grown in the presence of glucose in order to deplete the endogenous Isu1p, a significant amount of radioactive iron was co-immunoprecipitated with the mitochondrial F1β-hIsu2-HA protein (60% of the value obtained for the endogenous Isu1p). Because growth in the presence of glucose did not influence the levels of hIsu2-HA in this strain (Fig. 3*B*), these results indicate that the human protein can serve as an Fe/S cluster scaffold in yeast, but only in the absence of the endogenous yeast Isu1p. Apparently, yeast has a strong preference for its own Isu proteins. Strikingly, only background levels of radioactive iron were found associated with the cytosolic versions of human hIsu1-HA and Δ33-hIsu2-HA, even when Gal-ISU1/Δisu2 cells were cultivated in the presence of glucose (Fig. 3*A, left*). Because these human proteins were highly expressed (Fig. 3*B*), the data indicate that the cytosolic hIsu proteins were not capable of binding an Fe/S cluster in the yeast cytosol. This finding is in accordance with the observation that the cytosolic versions of the human Isu proteins did not rescue the growth defect of the Gal-ISU1/Δisu2 strain on glucose (26).

The observed defect in Fe/S cluster association on the cytosolic human Isu proteins may be because of a lack of a functional mitochondrial ISC machinery in the experiments described above. In order to explore this possibility, the cytosolic hIsu2-HA and Δ33-hIsu2-HA proteins were expressed in Gal-ISU1/Δisu2 cells that co-expressed an untagged version of F1β-hIsu2. The latter protein is localized exclusively in yeast mitochondria and rescues the growth defect of the Gal-ISU1/Δisu2 strain on glucose (26) but is not recognized by anti-HA antibodies. When these cells were radiolabeled with ^{55}Fe and the cytosolic Isu proteins hIsu2-HA and Δ33-hIsu2-HA were immunoprecipitated with anti-HA antibodies, only background levels of radioactive iron were found associated with the immunobeads (Fig. 3*A, right*). Moreover, the choice of the carbon source for growth did not affect ^{55}Fe binding to these proteins, indicating that the lack of Fe/S formation on the human cytosolic Isu proteins was not precluded by the more efficient Fe/S cluster formation on the endogenous yeast Isu proteins. Levels of the cytosolic hIsu proteins were similar to those of the mitochondrial protein F1β-hIsu2-HA in Gal-ISU1/Δisu2 cells (Fig. 3*B*). These results therefore unambiguously show that the human Isu proteins cannot assemble Fe/S clusters when located in the yeast cytosol, even in the presence of a functional mitochondrial ISC assembly machinery. Because the Isu proteins are the central scaffolds for *de novo* Fe/S cluster formation, the lack of Fe/S cluster formation on these proteins in the cytosol strongly suggests that the mitochondrial ISC machinery including Nfs1p is most likely not duplicated in the cytosol of *S. cerevisiae*.

Nfs1p Is Required for Thiolation of tRNA in S. cerevisiae—In bacteria, IscS has been demonstrated to be required for the formation of all thiouridine-modified tRNAs. A similar role for Nfs1p in thio-modification of tRNA could serve to explain the essential character of the nuclear Nfs1p species in *S. cerevisiae*. In order to explore this hypothesis, the incorporation of sulfur into tRNA was investigated. To this end, wild-type cells and Nfs1p-depleted Gal-NFS1 cells were radiolabeled with [^{35}S]methionine/cysteine, and total RNA was isolated. Subsequently, tRNA was separated by PAGE from the bulk of RNA and analyzed for the incorporation of radioactive sulfur. As shown in Fig. 4*A*, a significant amount of radioactivity was found associated with the tRNA fraction in wild-type cells. When total RNA was digested with RNase A and subjected to electrophoresis, the ^{35}S radiolabel of this fraction disappeared, indicating that the radioactivity found in this fraction in fact corresponded to tRNA (Fig. 4*B*). In contrast, almost no radioactive ^{35}S was found associated with this tRNA fraction in Nfs1p-depleted Gal-NFS1 cells (Fig. 4*A*). In order to determine whether these cells are still capable of tRNA synthesis, the tRNA fractions were assayed for the presence of the tRNA^{Lys} (UUU) in Northern blots. The amounts of tRNA^{Lys} (UUU) were even higher in Nfs1p-depleted Gal-NFS1 cells than in wild-type cells. These data indicate that the lack of ^{35}S radiolabeling of tRNA in the absence of Nfs1p was due to a lack of incorporation of sulfur into the tRNA moiety rather than due to a defect in tRNA synthesis. The impairment of sulfur incorporation into tRNA in Gal-NFS1 cells was specifically caused by the lack of Nfs1p, because the radiolabeling of tRNA remained unaffected in Nfs1p-depleted Gal-NFS1 cells that contained an extrachromosomal copy of *NFS1* (Fig. 4*C*).

The lack of tRNA modification in the absence of Nfs1p may indicate either a direct involvement of Nfs1p in sulfur insertion into tRNA or the requirement of Fe/S proteins in tRNA modification. In the latter case, the involvement of Nfs1p in tRNA modification would be a consequence of its role in Fe/S cluster maturation and thus only indirect. In order to distinguish these possibilities, the insertion of radioactive ^{35}S into tRNA was analyzed in the strains Gal-ATM1 and Gal-YAH1 which allow

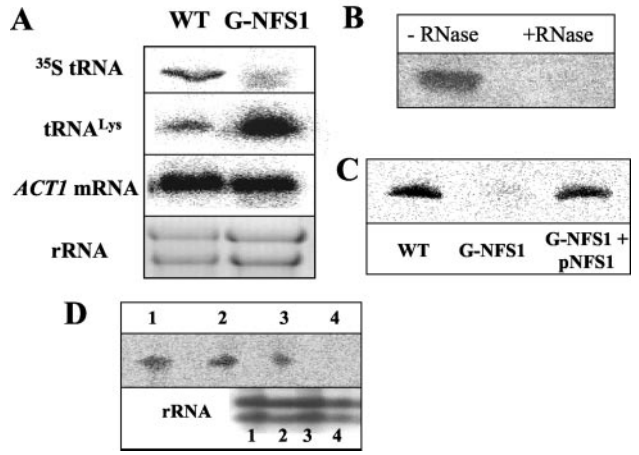

FIG. 4. **Nfs1p is essential for tRNA modification in *S. cerevisiae*.** *A*, wild-type (*WT*) and Gal-NFS1 cells (G-NFS1) grown in minimal medium supplemented with glucose were radiolabeled with [^{35}S]methionine/cysteine for 30 min. Total RNA was extracted and separated by denaturing PAGE, and ^{35}S-labeled tRNA was detected by autoradiography. tRNA^{Lys} (UUU) and the mRNA for actin (*ACT1*) were detected by Northern blot analysis. 25 S and 18 S rRNA were visualized by ethidium bromide staining in agarose gels and served as an input control. *B*, the total RNA fraction of ^{35}S-labeled wild-type cells was treated without or with RNase A and analyzed as above. *C*, wild-type and Gal-NFS1 cells lacking or harboring plasmid pNFS1 were radiolabeled with [^{35}S]methionine, and radiolabeled tRNA was determined by autoradiography as described above. *D*, wild-type (*lane 1*) and the conditional yeast strains Gal-YAH1 (*lane 2*), Gal-ATM1 (*lane 3*), and Gal-NFS1 (*lane 4*) were grown in minimal SD medium, and sulfur insertion into tRNA was determined as above. The *inset* shows the amount of 25 S and 18 S rRNA in the total RNA fraction isolated from the respective strains.

the conditional expression of *ATM1* and *YAH1*, two genes essential for the maturation of extramitochondrial Fe/S proteins (18, 19). As shown in Fig. 4*D*, the insertion of ^{35}S into tRNA was still possible upon depletion of Atm1p and Yah1p in the respective Gal mutants. These data indicate that thiolation of the bulk of tRNAs in yeast involves the participation of Nfs1p as a direct sulfur donor and does not require additional Fe/S proteins. However, these results do not exclude that the modification of certain species of tRNA may also involve so far unidentified Fe/S proteins.

The N Terminus of Mature Nfs1p Is Essential for Proper Enzymatic Function—Whereas the existence of Nfs1p species both inside and outside of mitochondria seems well established in *S. cerevisiae*, the sorting mechanism that underlies the multiple localization of the protein remains unknown. In human cells, a cytosolic version of Nfs1 was detected that was smaller than the mitochondrial form (41). This cytosolic isoform was suggested to be generated by alternative utilization of start codons resulting in an N-terminally truncated protein that lacks the mitochondrial targeting sequence. The cytosolic human Nfs1 was supposed to initiate at a methionine residue corresponding to Leu-102 of Nfs1p from *S. cerevisiae* (Phe-5 of NifS from *Thermotoga maritima* (4)) (Fig. 5*C*). Comparison with other NifS-like cysteine desulfurases showed that this predicted cytosolic human Nfs1 is 4–6 amino acid residues shorter than any bacterial version of NifS-like proteins (Fig. 5*C*). Conspicuously, a strictly conserved tyrosine residue (Tyr-101 of *S. cerevisiae* Nfs1p) is absent in cytosolic human Nfs1. This residue is part of a highly conserved N-terminal β-strand (S1 in the structure of NifS from *T. maritima*) that forms a parallel β-sheet with a β-strand (S12 of NifS from *T. maritima*) of the small domain that hosts the active cysteine (Fig. 5*A*) (4, 5). A closer inspection shows that amino acids Val-3 to Asp-6 (of *T. maritima* NifS) form specific hydrogen bonds with several residues of the small domain, such as Tyr-317 and Ser-352 (Fig. 5*B*). Simultaneously, Tyr-4 and Asp-6 form hydrogen bonds with the subsequent residues Asn-7, Ala-9, and Thr-10. The latter residues are part of a short loop that connects the small and the large (PLP-containing) domain of the protein. The large number of specific contact sites indicates that the immediate N terminus of cysteine desulfurases is involved in establishing a tight physical connection between the large and small domains of the protein (4, 5). Because there are only three physical connections between these domains, the crystal structures of NifS-like proteins suggest that the immediate N terminus likely plays a crucial role in maintaining the structural integrity of the protein.

So far, the cytosolic version of human Nfs1 is the only protein family member lacking this N-terminal segment. We therefore examined the importance of the N-terminal sequence of Nfs1p for its enzymatic function. First, we analyzed whether similarly shortened versions of Nfs1p from *S. cerevisiae* would be functional. To this end, we constructed two plasmid vectors, pF1β-F_{95}/L-NFS1 and pF1β-L_{102}-NFS1, which encode two N-terminally truncated versions of Nfs1p under the control of the strong *MET25* promoter (supplemental Table S1). The first version was truncated up to residue Phe-95 and was thus similar to bacterial versions of NifS-like proteins. The second was truncated up to residue Leu-102, which corresponds to the exact starting position of the cytosolic version of human Nfs1p. Both coding sequences were fused in-frame to the mitochondrial targeting sequence (residues 1–40) of the F_1β-subunit from *Neurospora crassa*. Vectors harboring these constructs and a plasmid encoding a wild-type Nfs1p were transformed into Gal-NFS1 cells. As shown in Fig. 6*A,* synthesis of the Nfs1p protein initiating at residue 95 complemented the growth defect of Gal-NFS1 cells on nonfermentable carbon sources. This indicates that the long N-terminal extension of *S. cerevisiae* Nfs1p is not essential for its function, even though the colony size of the cells was slightly smaller than that of Gal-NFS1 cells producing the wild-type version of Nfs1p. In contrast, no growth was observed with Gal-NFS1 cells synthesizing the Nfs1p version initiating at residue 102. This result unambiguously demonstrates that the N-terminal β-strand formed by residues 99–104 is essential for the function of *S. cerevisiae* Nfs1p in yeast.

In *E. coli*, deletion of the *NFS1* orthologue *iscS* results in severely reduced activities of Fe/S proteins and defects in the biosynthesis of thiamine and nicotinic acid (34). The biosynthetic pathways of both vitamins include sulfur insertion steps that require PLP-dependent cysteine desulfurases. In order to test the functional importance of the N-terminal β-strand of NifS-like proteins in *E. coli*, we constructed two pUC19-derived vectors, pM_{93}-NFS1 and pM_{102}-NFS1, that carry two N-terminally shortened versions of yeast Nfs1p under the control of the *lac* promoter. The first version starts with a methionine at residue 93, similar to the bacterial NifS-like proteins. The second initiates at residue 102, which exactly corresponds to the N terminus of the cytosolic version of human Nfs1p (supplemental Table S1). Both plasmids and a pUC19 plasmid that allowed the expression of the recombinant protein used for the *in vitro* studies outlined above were transformed into a ΔiscS strain from *E. coli* (34). As shown in Fig. 6*B*, the Nfs1p versions initiating at residues 68 and 93 fully rescued the small colony phenotype of the ΔiscS strain in rich medium. This demonstrates that yeast Nfs1p was capable of donating sulfur for Fe/S biogenesis as well as for thiamine and nicotinic acid production in *E. coli*. In contrast, the Nfs1p version that initiated at residue 102 was not functional in *E. coli*, because its expression did not significantly improve the growth of the ΔiscS strain,

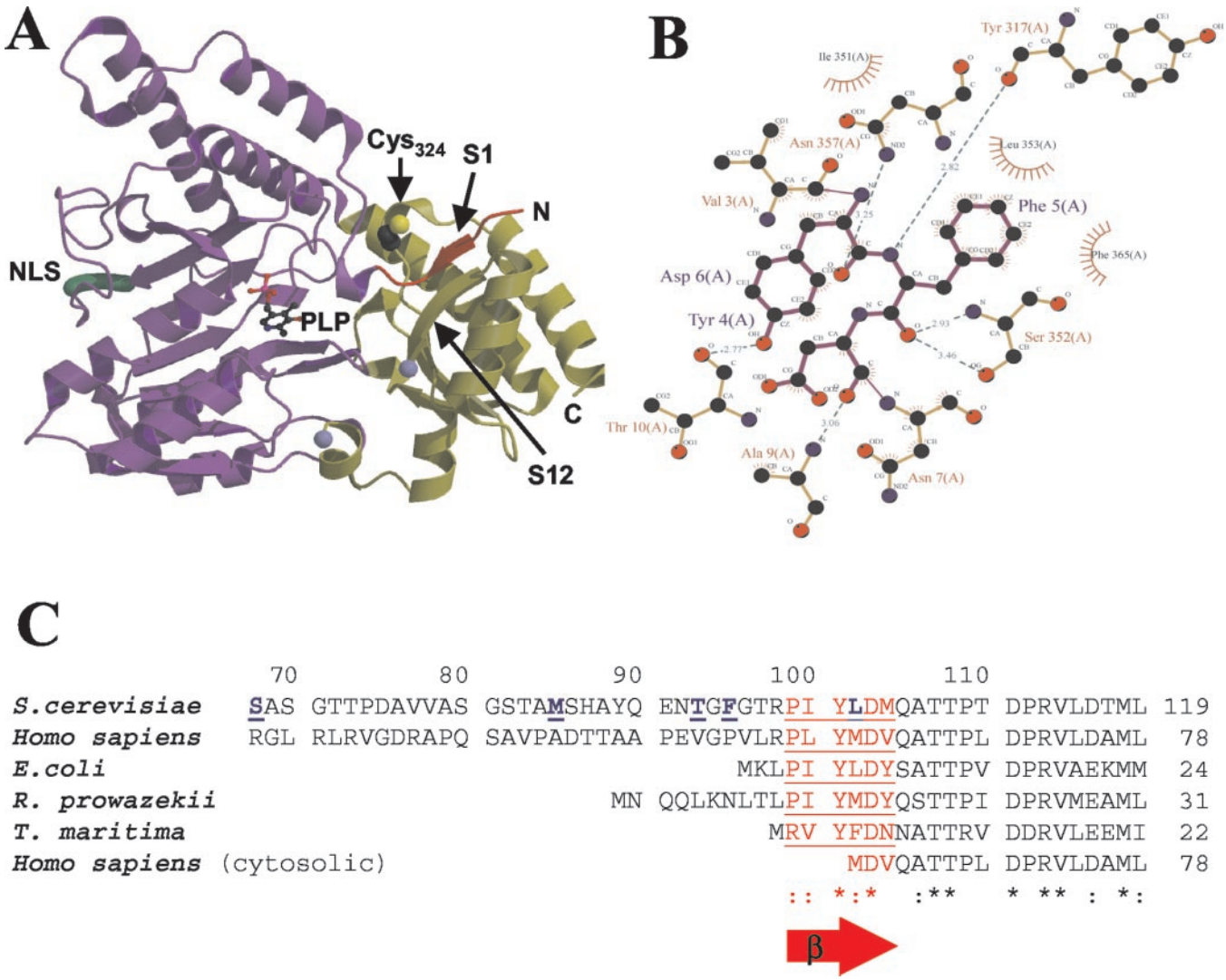

FIG. 5. **The N-terminal β-strand of bacterial cysteine desulfurases connects the two domains of the protein.** *A*, ribbon diagram of NifS from *T. maritima* (4). The N-terminal segment, displayed in *red*, forms a β-strand (*S1*). The active cysteine residue of the small domain is displayed in *yellow* and the potential nuclear targeting signal (*NLS*) in *green*; the pyridoxal phosphate is shown in *black* (for further details see Refs. 4 and 5). *B*, close-up of the protein environment around tyrosine 4 of *T. maritima* NifS. *Dashed lines* indicate intra-molecular hydrogen bonds. *C*, sequence alignment of the N-terminal regions of NifS-like proteins from the indicated species. The region forming the N-terminal β-strand S1 is indicated by a *red arrow*. Starting points of N-terminally shortened *NFS1* constructs used in this work are *underlined* and *boldface*. For details of these constructs see supplemental Table S1.

although the protein was detected by immunostaining (Fig. 6*C*). Taken together, these data demonstrate that the N-terminal β-strand of residues 99–104 is essential for the function of *S. cerevisiae* Nfs1p. Based on the structural importance of this segment, it is likely that this part is indispensable for the function of NifS-like cysteine desulfurases in general. These results are incompatible with the assumption of differential start codon utilization as a mechanism for the dual localization of human Nfs1.

DISCUSSION

In this work, we have carried out a detailed characterization of a eukaryotic NifS-like cysteine desulfurase. Recombinant Nfs1p from *S. cerevisiae* donates sulfur for Fe/S protein assembly using cysteine as a specific substrate *in vitro*. In *S. cerevisiae* and other eukaryotes, the majority of the protein is located within mitochondria (18, 41, 59). Here we show that this mitochondrial localization is both necessary and sufficient not only for the maturation of mitochondrial Fe/S but also for cytosolic and nuclear Fe/S proteins. Furthermore, we demonstrated that the human versions of the essential Fe/S scaffolding proteins hIsu1 and hIsu2 remain in their apoform, when located in the yeast cytosol. The lack of function of these essential ISC constituents strongly suggests that the maturation of extramitochondrial Fe/S proteins in *S. cerevisiae* does not involve cytosolic copies of the mitochondrial ISC components. By using ^{35}S radiolabeling of yeast cells *in vivo*, we detected a crucial role of Nfs1p in the thiolation of tRNA. tRNA modifications were also observed in yeast cells depleted for ISC proteins other than Nfs1p, indicating that Nfs1p is involved directly as a sulfur donor for tRNA thiolation. These results, which are in perfect agreement with recent data by Nakai *et al.* (44), convincingly explain the essential character of the extramitochondrial Nfs1p. The dual role of yeast Nfs1p is reminiscent of its bacterial homologue IscS. Finally, we assign an important role of an N-terminal β-strand of Nfs1p for the function of this protein. Most likely, this segment stabilizes the structure of the protein by serving as a linker connecting its two domains.

Functional studies in yeast have repeatedly shown that Fe/S cluster formation of cytosolic Fe/S proteins requires a contribution of mitochondria (reviewed in Refs. 14 and 15). Besides

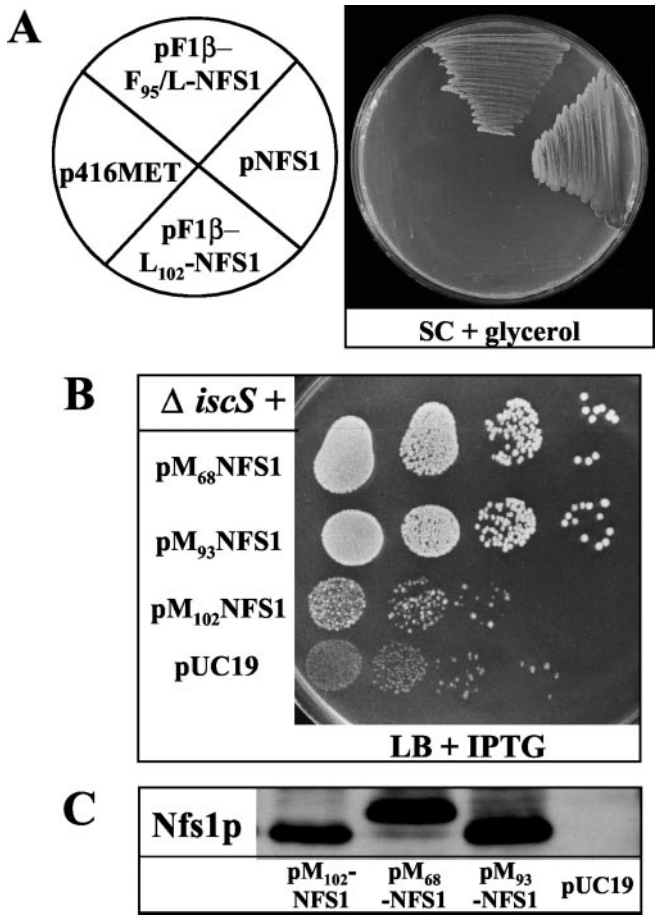

FIG. 6. **The N-terminal β-strand is essential for the function of Nfs1p from *S. cerevisiae*.** *A*, Gal-NFS1 cells harboring plasmid p416MET, a plasmid encoding either a full-length Nfs1p (pNFS1), or mitochondria-targeted versions starting at either residues 95 (pF1β-F_{95}/L-NFS1) or 102 (pF1β-L_{102}-NFS1) were grown on synthetic minimal medium (SC) supplemented with glycerol at 30 °C. *B*, *E. coli* ΔiscS cells were transformed with plasmids pM_{68}-NFS1, pM_{93}-NFS1, pM_{102}-NFS1, or pUC19. Cells were grown to $A_{600} = 0.5$, and serial dilutions of 10^{-4} to 10^{-7} were spotted on LB plates supplemented with kanamycin, ampicillin, and isopropyl-1-thio-β-D-galactopyranoside and incubated at 37 °C. *C*, immunostaining for Nfs1p levels in extracts from the *E. coli* ΔiscS strains shown in *B*. Plasmids used are described in the supplemental Table S1.

the mitochondrial ISC assembly machinery, the maturation of extramitochondrial Fe/S proteins requires the mitochondrial ISC export machinery, whose members include the mitochondrial ABC transporter Atm1p. The molecular details of the mechanism of cytosolic Fe/S protein assembly, however, has not been elucidated yet. Cell fractionation analyses in human cells have indicated the presence of small amounts of several of the mitochondrial ISC components in the cytosol, including Nfs1 (41, 58, 60). Hence, it was suggested that the mitochondrial ISC machinery is duplicated in the cytosol, where it may be required for the maturation of extramitochondrial Fe/S proteins. In *S. cerevisiae*, with the exception of Nfs1p, no evidence for the presence of any ISC components outside mitochondria has been provided previously. Our studies of differential targeting of Nfs1p to various cell compartments showed that disruption of its nuclear targeting signal did not destroy its enzymatic function as a sulfur donor in Fe/S protein maturation in mitochondria, cytosol, and nucleus. Hence, Fe/S protein maturation provides no valid explanation for the essential character of nuclear Nfs1p in yeast (43). As it was technically impossible to exclude residual targeting of Nfs1p to the cytosol, we employed the cytosolic forms of the human Isu scaffold proteins to test for the potential function of cytosolic Nfs1p. Our data provided no evidence for the functionality of the human Isu proteins as scaffolds for Fe/S cluster formation in the yeast cytosol. Only in mitochondria hIsu2 served as an efficient Fe/S cluster scaffold. We therefore conclude that Nfs1p is not functional in Fe/S cluster formation outside mitochondria. A similar conclusion was recently obtained for the yeast Isu proteins (26). In this case, even mis-targeting of Isu1p to the yeast cytosol was impossible. As a consequence, there appears to be no functional duplication of the mitochondrial ISC machinery in the cytosol, at least for *S. cerevisiae*. In this context, novel components required for Fe/S cluster maturation in the cytosol have been identified recently in yeast. The P-loop ATPase, Cfd1p, is essential for yeast viability and is highly conserved throughout eukaryotes, including man, but is unrelated to any known mitochondrial ISC protein (61). Recently, we have identified a second component of this cytosolic Fe/S protein assembly machinery, Nar1p (57). This protein is related to bacterial iron-only hydrogenases, and like Cfd1p, it is essential for viability and is highly conserved in eukaryotes. Taken together, these findings suggest that the cytosolic machinery required for extramitochondrial Fe/S cluster maturation in eukaryotes is distinct from the ISC machinery in mitochondria.

In bacteria, NifS-like cysteine desulfurases serve as versatile sulfur donors in several divergent biochemical pathways, such as Fe/S cluster biogenesis, vitamin synthesis, and tRNA modification. In eukaryotes, several lines of evidence point to the involvement of this class of apparently multipurpose enzymes in distinct but so far undefined metabolic pathways other than Fe/S protein biogenesis. *NFS1* was first identified as a suppressor of a tRNA splicing defect in yeast (62). Until now, no molecular explanation has been provided for this genetic observation. As found recently, the protein carries a potential nuclear targeting signal (43). Mutation of this signal in yeast results in loss of cell viability; however, the precise function of the extramitochondrial Nfs1p remained unknown. The involvement of Nfs1p in the thiolation of tRNA offers a simple and convincing explanation for the essential character of the extramitochondrial Nfs1p species. During the compilation of our manuscript, an important function of Nfs1p in thiouridine modification of both nuclear and mitochondrial tRNA was reported (44). Our data are in accordance with these observations. Our demonstration that the thiolation of tRNA was possible in cells deficient in cellular Fe/S protein maturation indicates that Nfs1p functions as a direct sulfur donor and that the failure of tRNA modification in the absence of Nfs1p is not a secondary consequence of its involvement in cellular Fe/S biogenesis. This dual role of yeast Nfs1p is reminiscent of its bacterial homologue IscS (34–36). Thiolation of the five thiolated bacterial tRNAs is thought to take five distinct routes, each involving specific enzymes that recognize a distinct subset of tRNAs (38). At least two of these routes involve Fe/S proteins, such as MiaB (39). In eukaryotes, little is known about thio-modifications of tRNA. Our data do not exclude that the modification of certain thiolated tRNA species may require so far unidentified Fe/S proteins. It has to be noted, however, that the pathways of tRNA thiolation in eukaryotes and bacteria differ considerably, as only one of the known bacterial tRNA-modifying enzymes, MnmA, has a clear homologue in *S. cerevisiae* (63). MnmA lacks conserved cysteine residues and thus in all likelihood is not an Fe/S protein.

In many eukaryotes, including *S. cerevisiae* and man, Nfs1p-like cysteine desulfurases are encoded by single genes. Hence, the sorting mechanism that is governing the simultaneous distribution of this protein into several subcellular compart-

ments is difficult to understand. The cytosolic Nfs1 species in human cells was claimed to be smaller than its mitochondrial version (41). This fact was explained by the utilization of an AUG start codon further downstream on the single mRNA transcript resulting in the formation of an N-terminally truncated protein that lacks the mitochondrial targeting sequence. Our data provide evidence against differential start codon utilization as an effective mechanism for sorting of Nfs1p in eukaryotes. The suggested cytosolic version of human Nfs1 would lack several residues that are highly conserved in all eukaryotic and bacterial NifS-like proteins. For Nfs1p from *S. cerevisiae*, truncation of these residues resulted in a complete loss of Nfs1p function in both yeast and bacteria. In addition, an AUG codon engineered at the same position as the human Nfs1 was not detectably used as a start codon in yeast (64). Moreover, many eukaryotic *NFS1* genes do not contain alternative start codons at their N termini. Based on these observations, it is reasonable to assume that the highly conserved N terminus of NifS-like proteins is likely indispensable for all proteins of this class, including Nfs1 from humans. The three-dimensional structures of NifS-like proteins provide the molecular explanation for this observation (4, 5). The conserved N-terminal stretch is part of a β-sheet structure that forms a physical linker between the two domains of this class of proteins. Most likely, this linker significantly contributes to the stability of NifS-like proteins.

Which mechanism might underlie the dual localization of Nfs1? Two different scenarios are possible. First, the pre-protein may be effectively routed to mitochondria, but a small portion may escape the import machinery by retrograde translocation into the cytosol after the presequence has been processed (65). Second, after translation a small portion of Nfs1p may be stably folded and assembled in the cytosol and may no longer be competent for import into mitochondria. The first scenario has been observed for fumarase in yeast, which is located both in mitochondria and the cytosol (66). This protein is encoded by a single gene. Its precursor is targeted to the mitochondria, and the presequence is processed by the mitochondrial matrix processing protease, but the majority leaves the mitochondrial import channel in a retrograde translocation to end up as a folded protein in the cytosol (67). Further studies are necessary to unravel the targeting mechanism of Nfs1p.

Taken together, our data provide strong evidence for distinct cellular roles of the mitochondrial and nuclear species of Nfs1p. Mitochondrial Nfs1p is required for the biogenesis of both mitochondrial and extramitochondrial Fe/S proteins. The fraction of the protein that resides in the nucleus is required as a sulfur donor for thiolated tRNA and plays no obvious role in cellular Fe/S cluster formation in yeast. The sum of our data renders the existence of a cytosolic version of the mitochondrial ISC assembly machinery unlikely, at least for *S. cerevisiae*. In fact, there is increasing evidence for the existence of an extramitochondrial Fe/S cluster maturation machinery in eukaryotes that is unrelated to the ISC assembly machinery in mitochondria. Most likely, more components of this novel biochemical machinery will be identified in the near future.

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