Binding of the Chaperone Jac1 Protein and Cysteine Desulfurase Nfs1 to the Iron-Sulfur Cluster Scaffold Isu Protein Is Mutually Exclusive*†‡§

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Biogenesis of mitochondrial iron-sulfur (Fe/S) cluster proteins requires the interaction of multiple proteins with the highly conserved 14-kDa scaffold protein Isu, on which clusters are built prior to their transfer to recipient proteins. For example, the assembly process requires the cysteine desulfurase Nfs1, which serves as the sulfur donor for cluster assembly. The transfer process requires Jac1, a J-protein Hsp70 cochaperone. We recently identified three residues on the surface of Jac1 that form a hydrophobic patch critical for interaction with Isu. The results of molecular modeling of the Isu1-Jac1 interaction, which was guided by these experimental data and structural/biophysical information available for bacterial homologs, predicted the importance of three hydrophobic residues forming a patch on the surface of Isu1 for interaction with Jac1. Using Isu variants having alterations in residues that form the hydrophobic patch on the surface of Isu, this prediction was experimentally validated by in vitro binding assays. In addition, Nfs1 was found to require the same hydrophobic residues of Isu for binding, as does Jac1, suggesting that Jac1 and Nfs1 binding is mutually exclusive. In support of this conclusion, Jac1 and Nfs1 compete for binding to Isu. Evolutionary analysis revealed that residues involved in these interactions are conserved and that they are critical residues for the biogenesis of Fe/S cluster protein in vivo. We propose that competition between Jac1 and Nfs1 for Isu binding plays an important role in transitioning the Fe/S cluster biogenesis machinery from the cluster assembly step to the Hsp70-mediated transfer of the Fe/S cluster to recipient proteins.

Iron-sulfur (Fe/S) clusters, ancient prosthetic groups found in all three domains of life, are present in a variety of proteins that function in essential cellular processes ranging from metabolism to the sensing of stress. In eukaryotic cells, mitochondria, which inherited their Fe/S cluster biogenesis system from bacterial ancestors, play a central role in maturation of cellular Fe/S cluster-containing proteins (1). Because the Saccharomyces cerevisiae Fe/S cluster biogenesis (ISC) system is the focus of this report, we use the gene/protein designation for this organism throughout. In the related bacterial and mitochondrial systems, the highly conserved small scaffold protein Isu serves as a platform on which a Fe/S cluster is assembled de novo prior to transfer to recipient proteins (1). In S. cerevisiae, Isu is encoded by a paralogous, functionally exchangeable gene pair, ISU1 and ISU2. Isu1 plays the major functional role because of its higher level of expression (2, 3). Both the assembly and the transfer steps require the interaction of Isu with other proteins.

Several lines of evidence from bacterial and mitochondrial systems indicate that a “Fe/S cluster assembly complex” constitutes a functional and structural unit responsible for de novo synthesis of a cluster on the scaffold (4–8). Most relevant to this report, Nfs1, a 51-kDa cysteine desulfurase, donates the sulfur needed for Fe/S cluster synthesis from cysteine, transferring it to Isu. In S. cerevisiae and other eukaryotes, Nfs1 is in a complex with the small accessory protein Isd11 (referred to as Nfs1(Isd11) throughout). Isd11 is proposed to both stabilize the Nfs1 protein and regulate its catalytic activity (9–11). The yeast frataxin homolog Yfh1, which in humans is associated with Friedreich’s ataxia, a neurological disease characterized by impairment of Fe/S cluster biogenesis and iron metabolism (12–14), is also part of the assembly complex. The function of

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Yfh1, which interacts with both Isu and Nfs1(Isd11), may be to serve as an iron donor and/or a regulator of cysteine desulfurase activity (4, 15, 16).

In both bacterial and mitochondrial ISC systems the Fe/S cluster transfer step is mediated by the J-protein-Hsp70 molecular chaperone system (17–19). A conserved specialized J-protein, called Jac1 in S. cerevisiae, is present in all eukaryotes and proteobacteria. As is typical in cases in which a J-protein also interacts with a client protein, the interaction of Jac1 with Isu is key, serving to target Hsp70 to its binding site on Isu (20–22). It is thought that conformational changes of the cluster-containing scaffold induced upon Hsp70 interaction triggers the release of the Fe/S cluster from the scaffold and its transfer onto a recipient protein (23). Strikingly, although most J-proteins that interact with client proteins display a rather broad specificity, interacting with a wide array of client proteins, the interaction of Jac1 with the client is very specific. Isu is its only known client (19, 20). In both the bacterial and mitochondrial systems, the C-terminal domain of Jac1 is directly responsible for Isu binding, with three hydrophobic residues playing a critical role in the interaction of Jac1 with Isu (22, 24, 25). Substitution of these residues with alanine sharply reduces the interaction of Jac1 with Isu in vitro and severely compromises both cell growth and the activity of the Fe/S cluster containing enzymes in vivo.

Not surprisingly, considering its central role in both the assembly and the transfer of Fe/S clusters, the Isu scaffold interacts with multiple other components of the Fe/S cluster biogenesis system (1). However, little information is available regarding the nature of these interactions and the functional consequences of their disruption. To begin to address these issues, we initiated a structure/function analysis. We identified three surface-exposed hydrophobic residues of Isu1 critical for its interaction with Jac1. These three residues were also critical for the interaction of Isu1 with the cysteine desulfurase Nfs1. Consistent with this dual role, Jac1 and Nfs1 competed with each other for Isu1 binding. On the basis of our experimental findings and the evolutionary conservation of residues involved in these protein-protein interactions, we hypothesize that the mutual exclusivity of these interactions plays a functional role in the transition between Fe/S cluster assembly and Fe/S cluster transfer steps in the biogenesis of Fe/S proteins.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids, Media, and Chemicals**—All strains were of the S. cerevisiae W303 background. The double mutant having deletions of both ISL1 and ISL2 (26) is referred to as isu-Δ throughout. To assess the level of the Nfs1 or Isu1 variants themselves or the activity of the Fe/S cluster containing enzymes, strains having the relevant WT gene at the normal chromosomal location under the control of the glucose-repressible GAL1–10 promoter were used (27). ISU1 and NFS1 mutants were generated in pRS314-ISU1 and pRS316-NFS1 using the Stratagene QuikChange protocol (26), as were all mutants in Escherichia coli expression vectors. JAC1 strains and plasmids have been described previously (22). Yeast was grown on YPD (1% yeast extract, 2% peptone and 2% glucose) or synthetic medium as described (28). All chemicals, unless stated otherwise, were purchased from Sigma.

**Protein Purification**—Nfs1(Isd11) was purified from _E. coli_ strain BL21-CodonPlus harboring plasmid pETDuet1-His-NFS1/ISD11. pETDuet1-HisNFS1/ISD11, which encodes a polyhistidine tag at the N terminus of Nfs1, was a gift from Dr. Roland Lill (Philips-Universität, Marburg, Germany). Expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside at A$_{600}$ = 0.6. During the 20 °C overnight induction period, cells were supplemented with 50 μM pyridoxal phosphate and 3% ethanol. Cells were harvested by centrifugation and lysed by French press in NA buffer (50 mM sodium phosphate (pH 6.5), 300 mM NaCl, 10% glycerol, 20 mM imidazole (pH 6.5), and 1 mM PMSF). After a clarifying spin, the yellow supernatant was loaded on a nickel-nitrilotriacetic acid column (Novagen) equilibrated with NA buffer (without PMSF). Proteins were eluted with a linear 20–250 mM imidazole gradient in NA buffer (12 column volumes). The rest of the yellow complex bound to the resin was eluted by a wash step with 400 mM imidazole in NA buffer. Fractions containing pure Nfs1(Isd11) complex were collected, pooled, and concentrated on a Centriprep 10K (Millipore). Highly concentrated complex was subjected to buffer exchange to buffer F (40 mM Heps-KOH (pH 7.5), 100 mM KCl, 1 mM dithiothreitol, 5% glycerol, and 10 mM MgCl$_2$) with a PD-10 column (GE Healthcare). Nfs1(Isd11) was aliquoted and stored at −70 °C.

Recombinant Jac1His WT and mutant proteins were also purified as described previously (20), except _E. coli_ strain C41(DE3) was used for expression. Recombinant Isu1-GST fusions were purified as described (22). In all cases, protein concentrations, determined using the Bradford (Bio-Rad) assay with bovine serum albumin as a standard, are expressed as the concentration of monomers.

**Pull-down Assay**—Titration pull-down experiments were performed by incubating the indicated concentrations of Jac1His or Nfs1(Isd11)$_{His}$ with 2.5 μM Isu1-GST in 150 μL of PD buffer (40 mM Heps-KOH (pH 7.5), 5% (v/v) glycerol, 100 mM KCl, 1 mM dithiothreitol, 10 mM MgCl$_2$, and 1 mM ATP) for 30 min at 25 °C to allow complex formation. Reduced glutathione-immobilized agarose beads were pre-equilibrated with 0.1% bovine serum albumin, 0.1% Triton X-100, and 10% (v/v) glyc erol in PD buffer. 40 μL of beads (~20–μL bead volume) were added to each reaction and incubated at 4 °C for 1 h with rotation. The beads were washed one time with 500 μL and then three times with 200 μL of PD buffer with 0.1% Triton X-100. Proteins bound to the beads were incubated with 2-fold-concentrated Laemmli sample buffer (20 μL) for 10 min at 90 °C, and 15-μL aliquots were loaded on SDS-PAGE and visualized by Coomassie staining.

**Cysteine Desulfurase Enzymatic Activity**—The enzymatic activity of Nfs1(Isd11) was measured as sulfide production using cysteine as the substrate according to Ref. 29. In the standard assay, 0.5 μM complex was incubated in 220 μL of CD buffer (20 mM Tris-HCl (pH 8.0), 200 mM sucrose, 50 mM NaCl, and 6 mM dithiothreitol) supplemented with 10 μM pyridoxal phosphate. The reaction was initiated by the addition of 0.5 mM l-cysteine. Following an incubation of 15 min at 25 °C, the reaction was terminated by the addition of 0.1 mL of 20 mM N,N-
dimethyl-p-phenylenediamine sulfate in 7.2 N HCl and 0.1 ml of 30 mM FeCl₃ in 1.2 N HCl. After further incubation in the dark for 20 min, the absorption of methylene blue was measured at 667 nm, and the sulfide concentration (nmol sulfide s⁻¹ per min per mg protein) was calculated on the basis of an Na₂S standard curve.

**Mitochondrial Enzyme Activities**—Activities of the respiratory enzymes were measured using mitochondria lysates as described previously in Ref. 30. Mitochondrial lysates were assayed for the activities of Fe/S cluster enzymes (succinate dehydrogenase and aconitase) and one non-Fe/S cluster protein (malate dehydrogenase) used here as a negative control. Succinate dehydrogenase activity was measured by using succinate as a substrate as described in Ref. 31. Aconitase activity was measured by monitoring the decrease in absorbance of the substrate isocitrate at 235 nm as described in Ref. 31. Malate dehydrogenase activity was measured using oxaloacetate as a substrate and by monitoring the decrease in absorbance of NADH at 340 nm as described in Ref. 31. Data were normalized to the protein content of the mitochondrial samples.

**Levels of Mitochondrial Proteins**—To quantify levels of Isu1 or Nfs1 variants, whole cell lysates were prepared by alkaline lysis (32) from 1 ml (A₆₀₀ = 1.0) of cell culture. The cell pellet was washed once with 0.5 ml of 10 mM Tris/HCl and 1 mM EDTA (pH 8.0) and then resuspended in 0.5 ml of cold H₂O. Cells were lysed by 10-min incubation after addition of 75 μl of freshly prepared 1.85 nL NaOH/7.4% 2-mercaptoethanol/10 mM PMSF. To precipitate protein, the mixture was incubated for 10 min on ice after addition of 575 μl of 50% trichloroacetic acid. After centrifugation, pellets were washed twice with 1 ml of ice-cold acetone prior to drying. Proteins were resuspended by incubation at 95 °C for 10 min after addition of 100 μl of Laemmli sample buffer. Insoluble material was removed by centrifugation, and proteins in the supernatant were separated in SDS-PAGE gels. The resolved proteins were transferred electrophoretically to nitrocellulose. Isu1 or Nfs1 variants were detected by enhanced chemiluminescence (33) using anti-Nfs1 or anti-Isu polyclonal antisemur.

**Prediction of Protein Structures**—The crystallographic structure of E. coli IscU from the IscS-IscU complex (PDB code 3LVL (7)) was selected by GenSilico Metaserver (34) as the best scored template for homology modeling of S. cerevisiae Isu1. A structure model was obtained using MODELLER (35) on the basis of alignments of the target protein sequence to the template structure prepared using MUSCLE (36). With the Isu1 structure model as a ligand and the crystallographic structure of S. cerevisiae Jac1 (PDB code 3UO3 (22)) as a receptor the computational docking procedure was performed using the ZDOCK server (37) with exclusion of the Jac1 J-domain from potential interaction. From the results obtained, the best-scored model of the Jac1-Isu1 complex was chosen for further optimization. Combination of manual and computational structural refinement was applied using DeepView-Swiss-PdbViewer (38) and Gaia (39), and short discrete molecular dynamics simulations were performed using Chiron (40). Of the optimized variants, the best-scored using FireDock (41) was chosen as the final model of the Jac1-Isu1 complex. Homology modeling of the S. cerevisiae Nfs1 structure was performed in the same way as Isu1, and the IscS from IscS-IscU (PDB code 3LVL (7)) was chosen as a structural template. Both Nfs1 and Isu1 models were overlaid with IscS-IscU crystallographic structure and, after an optimizing procedure the same as for the Jac1-Isu1 complex, a final model of the Nfs1-Isu1 complex was chosen as the best-scored using FireDock (41). Protein structure visualizations were prepared using the PyMOL Molecular Graphics System (version 1.5.0.4, Schrödinger, LLC).

**RESULTS AND DISCUSSION**

**Isu1 Residues Leu⁶³, Val⁷², and Phe⁹⁴ Are Critical for Jac1-Isu1 Interaction**—Previously (21, 22), we defined a binding interface consisting of eight hydrophobic and charged residues on the surface of the C-terminal domain of Jac1 involved in interaction with Isu1 (Fig. 1A). As a first step in defining the residues of Isu1 involved in interaction with Jac1, we performed in silico protein-protein docking simulations using the Jac1 structure (PDB code 3UO3 (22)) and a homology model of the Isu1 structure on the basis of the crystal structure of E. coli IscU (PDB code 3LVL (7)) because the structure of yeast Isu has not been determined. We chose ZDOCK predictions having the highest score for optimization to obtain the model of the Jac1-Isu1 complex presented in Fig. 1A. In this model, Jac1 hydrophobic and negatively charged residues interact with residues, hydrophobic and positively charged, respectively, on the surface of Isu1. More specifically, the hydrophobic region of Jac1 composed of Leu¹⁰⁵, Leu¹⁰⁹, and Tyr¹⁶³ interacts with three hydrophobic residues of Isu1, Leu⁶³, Val⁷², and Phe⁹⁴, whereas the charged region of Jac1 (Asp¹¹⁰, Asp¹¹³, and Glu¹¹⁴) interacts with a charged region of Isu1 encompassing residues Lys⁵⁴, Lys⁵⁵, and Arg⁷⁴. Strikingly, this interface is consistent with experimental data published previously for both yeast and bacterial systems (22, 24, 25, 44), although obtained independently. In particular, the hydrophobic residues (Leu¹⁰⁵, Leu¹⁰⁹, and Tyr¹⁶³) of Jac1 predicted by the modeling are the same as those found to be of critical importance for interaction with Isu1 in vitro studies of engineered variants (22). In addition, the Isu1-interacting residues obtained by modeling are among the residues of IscU, the E. coli Isu1 ortholog, for which chemical shift perturbations were observed by NMR spectroscopy upon addition of HscB (44), the Jac1 E. coli ortholog.

To test experimentally whether residues Leu⁶³, Val⁷², and Phe⁹⁴ of Isu1, predicted to contact these three residues, are important for interaction with Jac1, we constructed two ISU1
FIGURE 1. Replacement of the Leu<sup>63</sup>, Val<sup>72</sup>, and Phe<sup>94</sup> residues of Isu1 results in defective Jac1 binding and the inability to support cell growth. A, model of the Jac1-Isu1 complex (center panel) on the basis of in silico docking of the Jac1 protein crystal structure (PDB code 3U0S, left panel) and homology model of the Isu1 structure (right panel). Residues of Jac1 and Isu1 implicated in their interaction are highlighted. B, Isu1-GST-Jac1 pull-down. 2.5 μM Isu1-GST was mixed with the indicated concentrations of Jac1-WT, Jac1<sup>L63,V72,F94/AAA</sup> (LVF/AAA), or Jac1<sup>L63,V72,F94/SSS</sup> (LVF/SSS) and WT Jac1-GST with WT Isu1-GST (WT), Isu1<sup>L63,V72,F94/AAA</sup> (LVF/AAA), or Isu1<sup>L63,V72,F94/SSS</sup> (LVF/SSS) and WT Isu1-GST with WT Jac1 (WT), Jac1<sup>Y163/A</sup> (Y/A), or Jac1<sup>L105,L109,Y163/AAA</sup> (LLY/AAA). Glutathione resin was added to pull down the complex. Isu1-GST and Jac1 were separated by SDS-PAGE, visualized by staining, and quantitated by densitometry. Values were plotted in GraphPad Prism using a 1:1 binding hyperbola to fit data and plotted as relative units (r.u.), with maximum binding of WT protein given a value of 1. C, (left panel) jac1Δ cells harboring a plasmid-borne copy of WT JAC1 (on an URA3-based plasmid) and a second plasmid harboring WT ISU1 (WT), JAC1<sup>L63,V72,F94/AAA</sup> (LVF/AAA), or JAC1<sup>L63,V72,F94/SSS</sup> (LVF/SSS). Strains were plated on glucose-minimal medium containing 5-fluoroactic acid, which selects for cells having lost the plasmid containing the URA3 marker, and incubated at 30°C for 3 days. D, lysates of GAL-ISU1:isu2.D cells transformed with either a plasmid lacking an insert (-) or a WT copy of ISU1, ISU1<sup>L63,V72,F94/AAA</sup> or ISU1<sup>L63,V72,F94/SSS</sup>, under the control of the native ISU1 promoter, were prepared 17 h after transfer from galactose- to glucose-containing medium and separated by electrophoresis. Immunoblots were probed with antibodies specific to Isu1 and actin, a loading control.
cells with a plasmid carrying a mutant ISU1 allele under the control of the native ISU1 promoter, cultures were shifted from galactose- to glucose-based medium. WT Isu1 was depleted below the level of immunodetection, whereas the levels of both Isu1LVF/AAA and Isu1LVF/SSS were similar to that of Isu1 in a WT strain (Fig. 1D). We conclude that, despite normal expression levels, neither Isu1LVF/AAA nor Isu1LVF/SSS are able to support cell growth.

**Isu1 Residues Involved in Jac1 Interaction Are Important for Binding of Cysteine Desulfurase Nfs1**—The inability of Isu1LVF/AAA, which retained substantial affinity for Jac1, to support growth was surprising. Previously, we described a Jac1 variant, Jac1Y163/AAA, having an alteration on its Isu1 interaction surface that resulted in a similar reduction in Jac1-Isu1 interaction as that observed for Isu1LVF/AAA. However, Jac1Y163/A was able to support robust growth (22). We decided to reevaluate Jac1Y163/A (Fig. 1, B and C). We also included Jac1LLY/AAA in our analysis, which has alanine replacements of Leu105, Leu109, and Tyr163, the three hydrophobic residues found to be critically important for interaction with Isu1 in our earlier analysis (22). As expected, we found that Jac1LLY/AAA had negligible affinity for Isu1 (Fig. 1B) and only supported very slow growth (C). This contrast between growth phenotypes and Jac1-Isu1 affinities suggested that residues Leu63, Val72, and Phe94 of Isu1 may have a function(s) in addition to serving as an interface for interaction with Jac1. Inspection of structural data that recently became available for the bacterial orthologs of Isu and Nfs1 (IscU and IscS, respectively) is consistent with the idea that the Nfs1-Isu1 bind-domain includes the Isu1 residues Leu63, Val72, and Phe94 (7, 8, 45). To test whether these residues involved in the Jac1 interaction play a role in Nfs1 binding, we again used the Isu1-GST pull-down assay. When a fixed amount of Isu1-GST was incubated with increasing concentrations of purified Nfs1(Isd11), we observed that the amount of Nfs1 pulled down with Isu1-GST was concentration-dependent, with saturation reached at $\sim 10 \mu M$ Nfs1(Isd11) and half-maximal saturation at $\sim 1.5 \mu M$ Nfs1(Isd11) (Fig. 2). Thus, the affinity of Isu1 for Nfs1(Isd11) was similar to that observed for Jac1 (half-maximal saturation at $\sim 1 \mu M$ concentration, Fig. 1B).

To test whether residues Leu63, Val72, and Phe94 of Isu1 are indeed involved in the interaction with Nfs1(Isd11), we replaced these residues, either individually or in combination, with alanine. For Isu1L63/A-GST and Isu1V72/A-GST, we observed a substantial reduction of Nfs1(Isd11) binding in comparison to the Isu1-GST WT control (Fig. 2B). The effect of the Phe94/Ala replacement was less dramatic, with binding reduced by $\sim 25\%$. The ability of Isu1LVF/AAA-GST having the triple alanine substitution to bind Nfs1 (Isd11) was greatly reduced. Interaction of the LVF/SSS variant was less than 10% of the WT control, even at the highest concentration of Nfs1(Isd11) used in this experiment (Fig. 2B). These data indicate that the three residues of Isu1, Leu63, Val72, and Phe94, are critical for binding of both Jac1 and Nfs1(Isd11). Thus, a plausible explanation for the dramatic difference in phenotype of the jac1 and isu1 mutants (i.e. jacY/AAA and isu1LVF/AAA) resulting in partial disruption of the Jac1-Isu1 interaction is that the isu mutants have a more severe effect on Nfs1 binding.

**FIGURE 2. Replacement of residues Leu63, Val72, and Phe94 of Isu1 results in defective interaction with Nfs1(Isd11) in vitro.** A, Nfs1(Isd11), at the indicated concentrations, was mixed with 2.5 $\mu M$ Isu1-GST (left) or incubated in the absence of GST fusion (right). Glutathione resin was added to pull down the complex. Proteins were separated by electrophoresis and visualized by staining. B, top panel, schematic of variants tested. Bottom panel, Isu1-GST (2.5 $\mu M$) WT and variants, as indicated, were treated as described in A. Results were quantitated by densitometry, and obtained values were plotted in GraphPad Prism using a 1:1 binding hyperbola to fit data and plotted as relative units (r.u.), with WT Isu1 given a value of 1.

To further test the idea that the same residues of Isu interact with Jac1 and Nfs1, we decided to obtain a variant of Nfs1 defective in interaction with Isu1. We took advantage of structural information published previously about the complex of the bacterial orthologs (7, 8) to model the Nfs1-Isu1 complex. Three hydrophobic residues in the C-terminal region of Nfs1 (Pro478, Leu479, and Met482) were candidates for Isu1-interacting residues (Fig. 3A). Because alteration of a proline often leads to disruption of structural integrity, we choose to construct Nfs1 variants having alanine substituted at positions Leu479 or Met482 as well as in combination. We tested the ability of the purified Nfs1(Isd11) variants to interact with Isu1 using the Isu1-GST pull-down assay. The binding ability of both Nfs1L479/A(Isd11) and Nfs1M482/A(Isd11) variants was $\sim 50\%$ lower than that of WT protein, whereas binding of the variant having both alterations, Nfs1LM/AA(Isd11), was about 80% lower (Fig. 3B). We conclude that residues Leu479 and Met482 of Nfs1 are critical for interaction with Isu1. This conclusion is in accord with data indicating the importance of the C-terminal segment of both Nfs1 (46) and its E. coli ortholog (7) for interaction with the scaffold. We note that it is likely that residues in addition to Leu479 and Met482 play important roles in the interaction of Nfs1 with Isu1.
Overlapping Binding of Nfs1 and Jac1 to Isu

To assess the biological importance of the interaction of Nfs1 with Isu1 mediated by Leu<sup>79</sup> and Met<sup>82</sup>, we carried out in vivo experiments. We found that cells expressing Nfs1<sub>LM/AA</sub> were not viable (Fig. 3C) even though protein was expressed at the WT level. However, the cysteine desulfurase activity of Nfs1<sub>LM/AA</sub> was similar to that of the wild type (Fig. 3D), supporting the idea that these substitutions did not globally affect the structural properties of Nfs1. To ensure that the growth defects of the ISU1 and NFS1 mutants we observed were due to effects on Fe/S cluster biogenesis, we also tested the activity of two cluster-containing mitochondrial enzymes, aconitase and succinate dehydrogenase (SDH)<sup>5</sup>. As expected, the activity of aconitase and SDH was severely affected after depletion of WT Isu1 from cells expressing Isu1<sub>LVF/AAA</sub> (Fig. 3E) or after depletion of WT Nfs1 from cells expressing Nfs1<sub>LM/AA</sub> (F), consistent with the idea that direct interaction of Isu1 with Nfs1 is critical for the biogenesis of Fe/S cluster proteins.

*Competition between Nfs1 and Jac1 for Isu1 Binding*—Because the same three residues (Leu<sup>63</sup>, Val<sup>72</sup>, and Phe<sup>94</sup>) are important for the interaction of Isu1 with both Jac1 and Nfs1, we predicted that these two proteins directly compete for Isu1 binding (Fig. 4). To test this idea, we set up a biochemical competition assay on the basis of the Isu1-GST pull-down technique that we used to study the individual protein-protein interactions. First, we incubated a fixed amount of Isu1-GST with either Jac1 or Nfs1. Since Nfs1 and Jac1 compete with each other for binding to Isu1, Isu1-GST (2.5 \( \mu \)M) was mixed with either 5 \( \mu \)M Nfs1(Isd11) or 5 \( \mu \)M Jac1 to allow complex formation. Incubation was continued after addition of the indicated concentration of the competitor (Jac1, after preincubation with Nfs1(Isd11) (A) and Nfs1(Isd11) after preincubation with Jac1 (B)). Glutathione resin was then added to pull down Isu1-GST complexes. Proteins were separated by SDS-PAGE, visualized by Coomassie Blue staining, and quantitated by densitometry. Data were plotted as relative units (r.u.) with binding in the absence of a competitor given a value of 1.

**FIGURE 4.** Nfs1 and Jac1 compete with each other for binding to Isu1. Isu1-GST (2.5 \( \mu \)M) was mixed with either 5 \( \mu \)M Nfs1(Isd11) or 5 \( \mu \)M Jac1 to allow complex formation. Incubation was continued after addition of the indicated concentration of the competitor (Jac1, after preincubation with Nfs1(Isd11) (A) and Nfs1(Isd11) after preincubation with Jac1 (B)). Glutathione resin was then added to pull down Isu1-GST complexes. Proteins were separated by SDS-PAGE, visualized by Coomassie Blue staining, and quantitated by densitometry. Data were plotted as relative units (r.u.) with binding in the absence of a competitor given a value of 1.

5 The abbreviation used is: SDH, succinate dehydrogenase.

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**FIGURE 3.** Replacement of residues Leu<sup>79</sup> and Met<sup>82</sup> of Nfs1 results in reduced interaction with Isu1 and the inability to support cell growth. A, homology model of the Nfs1-Isu1 complex on the basis of the crystal structure of the complex of bacterial orthologous proteins (Iscs-IscU, PDB code 3LVL). Residues implicated in Nfs1-Isu1 interaction pertinent to this work are highlighted. B, Isu1-GST (2.5 \( \mu \)M), WT or variants, as indicated, were mixed with Nfs1(Isd11) at the indicated concentrations to allow complex formation. Glutathione resin was then added to pull down the complex. Proteins were separated by electrophoresis, visualized by staining, and quantitated by densitometry. Values were plotted in GraphPad Prism using a 1:1 binding hyperbola to fit data and plotted as relative units (r.u.). C, Nfs1 cells harboring aURA3-marked plasmid containing the WT NFS1 (WT) and a second plasmid harboring either WT NFS1 or nfs1<sup>L479,M482/AA</sup> (LM) were plated on glucose-minimal medium containing 5-fluoroorotic acid, which selects for cells having lost the plasmid containing galactose- to glucose-containing medium. As a standard, the enzymatic activity of non-Fe/S cluster-containing protein malate dehydrogenase (MDH) was measured. The ratio of activities of aconitase or SDH and malate dehydrogenase was calculated and expressed as a percentage of the WT control. Bars represent average values for three measurements, with presented error bars as S.D. D, aconitase activity and SDH activity were measured in lysates of mitochondria isolated from GAL-NFS1 cells harboring plasmid-borne copies of WT NFS1, nfs1LM/AA, or vector without insert, as indicated, grown for 40 h in glucose-containing medium. Enzymatic activities were measured and plotted as described in E.

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**FIGURE 4.** Nfs1 and Jac1 compete with each other for binding to Isu1. Isu1-GST (2.5 \( \mu \)M) was mixed with either 5 \( \mu \)M Nfs1(Isd11) or 5 \( \mu \)M Jac1 to allow complex formation. Incubation was continued after addition of the indicated concentration of the competitor (Jac1, after preincubation with Nfs1(Isd11) (A) and Nfs1(Isd11) after preincubation with Jac1 (B)). Glutathione resin was then added to pull down Isu1-GST complexes. Proteins were separated by SDS-PAGE, visualized by Coomassie Blue staining, and quantitated by densitometry. Data were plotted as relative units (r.u.) with binding in the absence of a competitor given a value of 1.
mixtures, followed by pull-down with glutathione resin. With an increasing concentration of Jac1, we observed a decreasing amount of Nfs1 associated with Isu1-GST so that, at 10 μM Jac1, the amount of Nfs1 in complex with Isu1-GST was reduced to < 50% of the initial value (Fig. 4A). When Jac1 WT was replaced by the Jac1LLY/AAA variant, which is strongly defective in Isu1 binding, no displacement of Nfs1(Isd11) associated with Isu1-GST was observed over a wide range of Jac1LLY/AAA concentrations. These results indicate that Jac1 and Nfs1 binding is mutually exclusive.

To verify this idea, we performed a reverse competition experiment. First, we incubated a fixed amount of Isu1-GST (2.5 μM) with a fixed amount of Jac1 (5 μM), allowing the formation of the Isu1-GST:Jac1 complex (Fig. 4B). Subsequently, either WT Nfs1(Isd11) or Nfs1L479,M482/AA(Isd11) was added to the reaction mixture. With increasing concentrations of WT Nfs1(Isd11), we observed decreasing amounts of Jac1 associated with Isu1-GST so that, at 5 μM Nfs1(Isd11), the amount of Jac1 in complex with Isu1-GST was reduced to ~50% of the initial value (Fig. 3B). However, no reduction in the amount of bound Jac1 was observed when Nfs1L479,M482/AA(Isd11) was added as a competitor, even when added at a concentration of 20 μM (Fig. 4B). This result is consistent with the idea that Leu479 and Met482 are indeed important for Nfs1-Isu1 interaction. Overall, we conclude that Nfs1 and Jac1 compete for overlapping binding sites on the surface of Isu1 that contain the three hydrophobic residues Leu63, Val72, and Phe94.

**Residues Involved in Jac1-Nfs1 Interactions with Isu1 Are Evolutionary Conserved**—The experiments described above were obtained using S. cerevisiae as the model system. To place our findings in an evolutionary context, we compared residues homologous to those involved in Nfs1/Jac1 interaction with Isu1 across the phylogeny. To this end, we identified orthologs of Jac1, Nfs1, and Isu1 from fully sequenced genomes of 84 eukaryotic species (62 fungal and 22 other eukaryotic species, including most model organisms) and 390 proteobacteria species (supplemental Table S1). Our analysis revealed that sites involved in Nfs1 and Jac1 binding are indeed evolutionarily conserved because they are either invariant across the phylogeny or they are occupied by highly similar amino acid residues (Fig. 5 and supplemental Table S1). For example, in only a limited number of eukaryotic Nfs1 orthologs, Met482 is replaced by leucine, and Tyr463 of the Jac1 orthologs is replaced by phenylalanine.
Interestingly, we also observed a pattern of residue conservation consistent with the hypothesis that \( \alpha \)-proteobacteria are closely related to ancestors of mitochondria because residues conserved in mitochondrial orthologs are identical to those of \( \alpha \)-proteobacteria, such as Rickettsia (47), and different from those of other bacterial species. For example, Leu\(^{63} \) and Phe\(^{94} \) of Isu1 are shared among mitochondrial and \( \alpha \)-proteobacteria orthologs, whereas orthologs from other proteobacteria species have mostly methionine and tyrosine at homologous positions. Overall, phylogenetic analysis revealed that the molecular mechanism of Nfs1-Jac1 interactions with Isu1 is evolutionary conserved and that mitochondrial proteins inherited residues involved in those interactions from their \( \alpha \)-proteobacterial ancestor.

**CONCLUSIONS**

Several lines of evidence indicate that the cysteine desulphurase Nfs1 and the J-protein cochaperone Jac1 bind to overlapping sites on Isu1, each containing the hydrophobic residues Leu\(^{63} \), Val\(^{72} \), and Phe\(^{94} \). These highly conserved overlapping sites render their interactions with Isu1 mutually exclusive. A central issue in Fe/S cluster biogenesis via the ISC pathway is understanding the transition from the process of assembly of the Fe/S cluster on Isu to the process of transfer (Fig. 6A). It is well accepted that Hsp70 is critical for cluster transfer and that the Jac1-Isu1 interaction is critical for targeting Isu1 for Hsp70 binding (1, 19, 48). The results reported here, indicating the exclusive nature of the Jac1 and Nfs1 interaction with Isu1, suggest that an ordered transition occurs from cluster assembly to cluster transfer.

What could be the driving force(s) behind such an ordered transition? Two factors seem most likely to play a role. First, the relative affinities of Nfs1 and Jac1 may differ for the apo- and holoforms of the scaffold (6, 49). Higher affinity for the apoform of Isu1 for Nfs1 (and holoforms of the scaffold (6, 49). Higher affinity for the apoform of Isu1 for Nfs1 (and holoforms of the scaffold (6, 49). Higher affinity for the apoform of Isu1 for Nfs1 (and holoforms of the scaffold (6, 49).

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