Mitochondrial biosynthesis of iron-sulfur clusters (ISCs) is a vital process involving the delivery of elemental iron and sulfur to a scaffold protein via molecular interactions that are still poorly defined. Analysis of highly conserved components of the yeast ISC assembly machinery shows that the iron-chaperone, Yfh1, and the sulfur-donor complex, Nfs1-Isd11, directly bind to each other. This interaction is mediated by direct Yfh1-Isd11 contacts. Moreover, both Yfh1 and Nfs1-Isd11 can directly bind to the scaffold, Isu1. Binding of Yfh1 to Nfs1-Isd11 or Isu1 requires oligomerization of Yfh1 and can occur in an iron-independent manner. However, more stable contacts are formed when Yfh1 oligomerization is normally coupled with the binding and oxidation of Fe^{2+}. Our observations challenge the view that iron delivery for ISC synthesis is mediated by Fe^{2+}-loaded monomeric Yfh1. Rather, we find that the iron oxidation-driven oligomerization of Yfh1 promotes the assembly of stable multi-component complexes in which the iron donor and the sulfur donor simultaneously interact with each other as well as with the scaffold. Moreover, the ability to store ferric iron enables oligomeric Yfh1 to adjust iron release depending on the presence of Isu1 and the availability of elemental sulfur and reducing equivalents. In contrast, the use of anaerobic conditions that prevent Yfh1 oligomerization results in inhibition of ISC assembly on Isu1. These findings suggest that iron-dependent oligomerization is a mechanism by which the iron donor promotes assembly of the core machinery for mitochondrial ISC synthesis.

ISC biosynthesis is an essential function that eukaryotic cells initiate in mitochondria and probably other cellular compartments using three core components: a sulfur donor, an iron donor, and an ISC assembly scaffold (1, 2). In yeast mitochondria, the cysteine-desulfurase, Nfs1, and the iron-chaperone, Yfh1, are believed to provide sulfur and iron, respectively, for ISC assembly on the Isu1 scaffold (1), whereas the Nfs1-binding protein, Isd11, has been shown to stabilize Nfs1 (3). These components are highly conserved and the human orthologues of Yfh1 (frataxin), Isu1 (ISCU), and Isd11 (ISD11) are implicated in the etiology of severe disorders including Friedreich ataxia and mitochondrial myopathy (4).

Previous studies have underscored the complexity of the interactions among eukaryotic ISC assembly components as well as their metal dependence. Supplementation of mitochondrial lysates with Fe^{2+} under aerobic conditions led to co-isolation of Yfh1 and Isu1 along with Nfs1 and Isd11 by pulldown or immunoprecipitation assays (5–7). Furthermore, aerobic preincubation of histidine-tagged Yfh1 monomer with Fe^{2+} enabled Isu1 to be pulled down by Yfh1 in the absence of other proteins (5). These studies have led to the current view that iron delivery for yeast ISC synthesis involves direct contacts between iron-loaded monomeric Yfh1 and Isu1 (5–7). Although Yfh1 oligomerization is normally coupled with iron binding, oxidation, and storage (5, 8), the possibility that Isu1 might also interact with oligomeric Yfh1 has remained largely unexplored.

Similar to Yfh1, human frataxin was found to interact with multiple ISC assembly components in human cells; however, in this case immunoprecipitation data suggested that frataxin binds to ISCU indirectly, via nickel-dependent contacts with ISD11 (9). Whether direct interactions occur between Yfh1 and Isd11 has not yet been examined.

While previous studies focused primarily on Yfh1-Isu1 and frataxin-ISD11 interactions, it is likely that the coordinate delivery of potentially toxic sulfur and iron to Isu1/ISCU involves multiple close interactions whereby the sulfur donor and the iron donor simultaneously interact with each other and with the ISC scaffold, as proposed for prokaryotic ISC assembly (10). However, it is currently unknown whether monomeric Yfh1/frataxin may form direct contacts with more than one partner, and the structure of the eukaryotic ISC assembly machinery is completely undefined. We show that iron oxidation-dependent oligomerization enables Yfh1 to have simultaneous direct interactions with Nfs1-Isd11 and Isu1. Our data provide insights about the sequence of events and the molecular architecture required for the initial step in mitochondrial ISC assembly.

**EXPERIMENTAL PROCEDURES**

Protein Expression and Purification—Yfh1 monomer, trimer, and 24-mer were expressed in *Escherichia coli* and purified as described (11). For expression of Isu1-His a DNA fragment corresponding to the predicted mature form of Isu1 (residues...
Core Machinery for Mitochondrial Fe-S Cluster Synthesis

28–166) (12) was cloned in vector pET28b (Novagen) in two steps: first, the fragment was cloned into the EcoRI and HindIII sites of pET28b, and second, an Ncol-EcoRI fragment was removed. This resulted in the addition of seven amino acids plus a six-histidine tag to the Isd11 C-terminus. Isd11His was expressed in E. coli strain BL21 and purified by nickel affinity chromatography (5-ml HiTrap Ni-column, Amersham Biosciences) followed by preparative gel-filtration chromatography (16-mm × 50-cm column packed with Superdex 75 support from Amersham Biosciences) essentially as described for Haemophilus influenzae IscU (13). Briefly, cells were grown at 25 °C in 1 liter of Luria broth containing 30 μg/ml kanamycin. Protein expression was induced at A600 = 0.6 with 0.5 mM isopropyl-β-D-thiogalactopyranoside, and growth continued at 15 °C for ~18 h. Cells were harvested, disrupted by sonication, and centrifuged at 25,000 × g for 20 min at 4 °C, and the soluble fraction was loaded onto a 5-ml HiTrap nickel column. The Nfs1-Isd11His complex was eluted in lysis buffer without protease inhibitors between 150 and 250 mM imidazole and was further purified by preparative gel-filtration chromatography (16-mm × 50-cm column packed with Superdex 200 support from Amersham Biosciences) in 10 mM HEPES-KOH, pH 7.8, 150 mM NaCl, 50 μM pyridoxal phosphate. The Nfs1-Isd11His complex was stable in this buffer at a protein concentration of ≤5 mg/ml.

A DNA fragment corresponding to the predicted mature form of Isd11 was cloned in the Ndel and HindIII sites of pET28b resulting in the addition of an N-terminal 6-histidine tag followed by a thrombin cleavage site as described above. For expression of Isd11His we used the same procedure employed for co-expression of Nfs1-Isd11His, except that induction with isopropyl-β-D-thiogalactopyranoside was for 2 h at 37 °C, because this resulted in a higher protein yield relative to other conditions tested. Isd11His was expressed at very low levels and exhibited a strong tendency to precipitate out of solution. Therefore, upon HiTrap nickel-affinity chromatography, fractions containing mostly Isd11His monomer were pooled and concentrated to ~0.4 mg of protein/ml in 10 mM HEPES-KOH, pH 7.8, 150 mM NaCl.

As determined by SDS-PAGE, Yfh1, Isu1His, untagged Isu1, and the Nfs1-Isd11His complex exhibited >90% purity, whereas isolated Isd11His exhibited >80% purity (supplemental Fig. S1). Protein concentration was determined by use of the BCA kit (Pierce) and is expressed in all cases per subunit. Iron content of purified proteins was measured as described (8).

In Vitro Pulldown Assays—Nickel-nitrotriacetic acid (Ni-NTA)-agarose (Qiagen) affinity pulldown experiments were started by incubating increasing concentrations of the appropriate His-tagged protein (Isu1His or Nfs1-Isd11His) with a fixed concentration (typically 4 μM) of each partner protein (Yfh1 and/or Isu1). Unless otherwise stated, binding was allowed to occur at 30 °C for 30 min in 100 μl of binding buffer (10 mM HEPES-KOH, pH 7.3, 100 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol). Stock solutions of ferrous ammonium sulfate (4–10 mM, pH ~4) were freshly prepared in water previously deaerated by purging with argon gas (<0.2 ppm O2). To generate holo-Yfh1 proteins, each apoform (typically 40 μM) was aerobically incubated with 10 equivalents of Fe(NH4)2(SO4)2 for ~15 h in 10 mM HEPES-KOH, pH 7.3, at room temperature on a nutator mixer, and added to the binding reaction to achieve the appropriate final concentration. The Fe2+/subunit molar ratio used to generate holo-Yfh1 proteins was lower than the maximum loading capacities of both Yfh1 and Yfh1-Y73A (50 and ~50 atoms of iron/subunit) (16, 17). In addition, iron loading was carried out under established aerobic conditions that lead to formation of a stable ferric iron mineral inside Yfh1 or Yfh1-Y73A oligomers (16, 18). Accordingly, ultrafiltration analysis of holo-Yfh1 forms in Ultrace-0.5 devices (Millipore) (8, 19) showed that ≥96.5% of the total iron was protein-bound (≥38 μM), whereas soluble free iron was ≤0.35 μM and insoluble iron was ≤2 μM. With holoproteins subjected to ultrafiltration, the absolute amounts of all three Yfh1 forms pulled down by Isu1His were not significantly different as compared with untreated holoforms (see Fig. 1D), and therefore ultrafiltration was omitted from subsequent experiments using holo-Yfh1 forms. Beads were washed and equilibrated with one volume of binding buffer containing
0.01% bovine serum albumin. Equilibrated beads (20 μl of a slurry containing equal volumes of beads and buffer) were added to each binding reaction and washed four times with 1 ml of binding buffer containing 0.1% Triton X-100. Bound proteins were eluted by boiling the beads for 5 min in Laemmli buffer and resolved by SDS-PAGE on Criterion precast Tris-HCl gel (Bio-Rad). The flow-through of each binding reaction was also analyzed on a separate gel as a control. This demonstrated that essentially 100% of the Isu1His or Nfs1-Isd11His added to the reactions was bound to the Ni-NTA beads. Gels were stained with SYPRO Orange (Invitrogen), and bound protein was quantified relative to the internal standard using ImageQuant 5.0 on a Storm 860 (Amersham Biosciences).

In Vitro Gel-filtration Chromatography Assays—Unless otherwise stated, samples (typically 250 μl) containing one or more of the proteins under investigation (typically 40 μM each protein) were incubated in binding buffer for 30 min at 30 °C. The binding reaction was centrifuged for 5 min at 20,000 × g and loaded onto the appropriate column equilibrated with the same buffer. Fractions were collected, and aliquots were analyzed by SDS-PAGE and SYPRO Orange staining. To generate holo-Yfh1 proteins, each apoform (typically 80 μM) was aerobically incubated with 10 equivalents of Fe(NH4)2(SO4)2 as described above and added to the binding reaction to achieve the required final concentration.

Iron-Sulfur Cluster Assembly Assay—ApO-α or apo-α24Y73A was aerobically incubated with Fe(NH4)2(SO4)2 at a Fe2+/subunit ratio of 1:2 for 75 min in 20 mM HEPES-KOH, pH 7.3, 150 mM NaCl (19), in parallel with samples containing the same iron concentration but no Yfh1. Ultrafiltration analysis (8, 19) showed that, in both the holo-α and holo-α24Y73A preparations, protein-bound iron was ≥46.5 μM (≥93% of the total 50 μM iron added to each protein), whereas free and insoluble iron were ≤2 μM and ≤2.5 μM, respectively. To verify the extent of iron oxidation, each holo-Yfh1 form was centrifuged at 20,000 × g for 10 min to eliminate insoluble iron, and the supernatant incubated with the Fe2+ chelator, α-α'-bipyridine (8, 19), which showed that ≥95.5% of the protein-bound iron was present in ferric form as expected. All buffers and solutions were purged with argon gas (<0.2 ppm O2) in vials tightly sealed with rubber septa. Each reaction was assembled in a quartz cuvette that was purged with nitrogen gas before and after addition of each component, and finally sealed with a tight-fitting rubber septum. [Fe2-2S] synthesis was started with addition of 50 μM iron (80 μl), provided as holo-α or holo-α24Y73A or directly, to anaerobic reactions containing 6.25 μM Isu1His, 2.5 mM Na2S, 5 mM dithiothreitol, and 2 mM l-cysteine in 20 mM HEPES-KOH, pH 7.3, 150 mM NaCl (800-μl final reaction volume). Reactions containing 0.5–1.0 μM Nfs1-Isd11His instead of Na2S were set up in a similar manner. Reactions containing holo-α(Fe2+) were similarly set up except that apo-α was incubated with Fe(NH4)2(SO4)2 anaerobically (8). ISC synthesis was monitored spectrophotometrically at A326.

In Vivo Pulldown Assays—Strains expressing wild-type and mutant Yfh1 proteins were described previously (17, 20). In all cases, cells were incubated at 30 °C in YPGal medium (1% yeast extract, 2% peptone, 2% galactose, 0.5% glucose). Cell growth and mitochondria isolation were performed as described (17). Mitochondria (800 μl at ~12 mg of total protein/ml) were suspended in 10 mM HEPES-KOH, pH 7.3, 100 mM NaCl, and disrupted by sonication, followed by treatment with 0.5% Triton X-100 for 30 min on ice. The mitochondrial lysate was cleared by centrifugation at 20,000 × g for 10 min at 4°C, and the supernatant (~4.5 mg of total protein) was used for the experiment. Pulldown assays were performed with Ni-NTA-agarose beads (40 μl slurry) with or without bound Isu1His or Nfs1-Isd11His, which were added to the mitochondrial lysate described above in a final 1-ml volume of 10 mM HEPES-KOH, pH 7.3, 100 mM NaCl, 20 mM imidazole, 0.05% Triton X-100. The final concentration of Isu1His or Nfs1-Isd11His was 0.8 μM. Binding reactions were incubated for 2 h at 4 °C on a nutator mixer. The agarose beads were washed three times in 800 μl of buffer and resuspended in 40 μl of Laemmli buffer after the last wash. Polyclonal antibodies were raised in rabbits using purified recombinant proteins as antigens.

RESULTS

Stable Yfh1-Isu1 Interactions Require Yfh1 Oligomerization—We postulated that the iron dependence of Yfh1-Isu1 interactions (5–7) might reflect stepwise assembly of Yfh1 monomer into trimer, 24-mer, and higher order oligomers, which occurs in an iron-dependent manner both in vitro (5, 21) and in yeast (17). We produced recombinant forms of Yfh1, Isu1, Nfs1-Isd11, and Isd11 in E. coli (supplemental Fig. S1). To distinguish whether Yfh1 interacts with its partners in an iron- or oligomerization-dependent manner, we used iron-free wild-type Yfh1 monomer (α) as well as stable iron-free trimer (α3Y73A) and 24-mer (α24Y73A) produced during expression of the Yfh1-Y73A variant in E. coli (11). The Y73A mutation stabilizes an assembly-competent conformation of Yfh1 and thereby accelerates its assembly kinetics both in E. coli and in yeast without changing its iron-chaperone function (17).

We initially tested binding of iron-free (apo) Yfh1 proteins to Isu1His by use of a pulldown assay, a method used in previous studies of Yfh1-Isu1 interactions (5, 7). Binding to Isu1His was much stronger with apo-α24Y73A protein than with apo-α or apo-α3Y73A (Fig. 1, A and B). The amounts of Yfh1 pulled down by Isu1His were not simply proportional to the number of Yfh1 subunits in monomer, trimer, and 24-mer because we obtained essentially identical results with apo-α and apo-α3Y73A. Moreover, each of the three protein preparations contained ≥0.02 atoms of iron per Yfh1 subunit, and the binding of apo-α24Y73A to Isu1His was only modestly decreased by EDTA (not shown), indicating that the interaction of Isu1His with apo-α24Y73A was mostly metal-independent. Next, α and α3Y73A were preincubated with Fe2+ aerobically under established conditions that induce stepwise assembly of monomer and trimer into 24-mer, and promote conversion of Fe2+ to Fe3+ inside Yfh1 oligomers via ferroxidase activity and autoxidation (8, 16). Binding of holo-α or holo-α3Y73A to Isu1His was stronger relative to the respective apo form and approached that of holo-α24Y73A (Fig. 1, C and D). We hypothesized that these results might reflect the presence of different levels of 24-mer in the α, α3Y73A and α24Y73A preparations before and after iron loading. To assess this, we analyzed Yfh1-Isu1His interactions in solution by gel-filtration chromatography.
Isu1\textsubscript{His} was eluted in fractions with apparent molecular mass \textasciitilde 44–17 kDa (Fig. 2A). An untagged version of Isu1 exhibited a very similar elution profile (supplemental Fig. S1d). The elution profiles of the apo-\(\alpha\), \(-\alpha\textsubscript{Y73A}\), and \(-\alpha\textsubscript{Y73A}\) proteins showed that they contained primarily Yfh1 monomer, trimer, and 24-mer, respectively, although additional species were present in each preparation (Fig. 2 (B and F) and supplemental Fig. S2b).

The distribution of Isu1\textsubscript{His} remained mostly unchanged upon incubation with apo-\(\alpha\) or apo-\(\alpha\textsubscript{Y73A}\) (Fig. 2C and supplemental Fig. S2c); in contrast, a significant proportion of Isu1\textsubscript{His} was co-eluted with 24-mer upon incubation with apo-\(\alpha\textsubscript{Y73A}\) (Fig. 2G). When apo-\(\alpha\) and apo-\(\alpha\textsubscript{Y73A}\) were preincubated with iron, they underwent iron-dependent oligomerization (Fig. 2D and supplemental Fig. S2d), and upon incubation with these proteins, most Isu1\textsubscript{His} was co-eluted with the newly assembled 24-mer (Fig. 2E and supplemental Fig. S2e). The co-elution profiles were almost indistinguishable from that obtained upon incubation of Isu1\textsubscript{His} with holo-\(\alpha\textsubscript{Y73A}\) (Fig. 2I).

We verified that the distribution of Isu1\textsubscript{His} remained unchanged when the protein was directly incubated with iron (not provided as holo-Yfh1; supplemental Fig. S3). We also verified that untagged Isu1 exhibited very similar interactions as Isu1\textsubscript{His} upon incubation with the \(\alpha\) preparation or \(\alpha\textsubscript{Y73A}\) preparation before and after iron loading (supplemental Fig. S4, a–g).

Samples containing equivalent concentrations of Yfh1 (provided as holo-\(\alpha\textsubscript{Y73A}\)), and untagged Isu1 were analyzed by Sephacryl 300 gel-filtration chromatography (fractionation range 1.5 MDa to 10 kDa). We observed a symmetrical peak with a molecular mass of \textasciitilde 400 kDa, and once again SDS-PAGE showed co-elution of 24-mer and Isu1 (Fig. 3). Together, these results indicate that Isu1 interactions with Yfh1 are initiated by Yfh1 oligomerization, which in the case of wild-type Yfh1 occurs concurrently with iron binding and oxidation.
Nfs1-Isd11 Complex Directly Interacts with Oligomeric Yfh1—Binding of Yfh1 proteins to Nfs1-Isd11_His complex was also examined by pulldown assay. Low amounts of apo-α and larger amounts of apo-α_{24}^{Y73A} were bound to the Nfs1-Isd11_His complex (Fig. 4, A and B). Preloading the Yfh1 proteins with iron resulted in an increased affinity of holo-α for Nfs1-Isd11_His to a level comparable to that of holo-α_{24}^{Y73A} (Fig. 4, A and B). Similar results were obtained with isolated Isd11_His (Fig. 4C), suggesting that the interactions observed above were mediated at least in part by Yfh1-Isd11 contacts. Isolated Nfs1 was not available to test for direct Yfh1-Nfs1 interactions.

Yfh1 (provided as holo-α) and untagged Isu1 could bind to Nfs1-Isd11_His, both separately and at the same time; notably, the amount of Yfh1 that was bound to Nfs1-Isd11_His did not change significantly in the absence or presence of Isu1 (Fig. 4D), suggesting that different surfaces of oligomeric Yfh1 are involved in interactions with Nfs1-Isd11_His and Isu1, respectively.

Iron-dependent Oligomerization of Yfh1 Promotes Formation of Stable Multicomponent Complexes—Equivalent concentrations of Isu1_His and Nfs1-Isd11_His were incubated in the absence or presence of equivalent amounts of Yfh1 (as apo or holo α or α_{24}^{Y73A}), and interactions analyzed in solution by Sephacryl 300 gel-filtration chromatography. Isu1_His was used in these experiments because it was less prone to degradation than untagged Isu1 (supplemental Fig. S1, a–d).

Low levels of Isu1_His were co-eluted with Nfs1-Isd11_His, whereas the bulk of Isu1_His was eluted in fractions between 44 and 17 kDa (Fig. 5A, fractions 72–84), as observed earlier by Superdex 75 gel-filtration chromatography (Fig. 2A). Nfs1-Isd11_His and the associated Isu1_His were eluted around ~300 kDa (Fig. 5A, fraction S8). The elution profiles of Nfs1-Isd11 and Isu1_His remained essentially unchanged when these proteins were incubated with apo-α (Fig. 5B), consistent with the inability of monomeric Yfh1 to form stable interactions with either component, as described above. Upon incubation with apo-α_{24}^{Y73A}, low levels of Nfs1-Isd11_His and Isu1_His were otherwise shifted to higher molecular weight fractions and were co-eluted with 24-mer (Fig. 5D, fractions 44–50). However, smaller proportions of Isu1_His and Nfs1-Isd11_His were associated with apo-α_{24}^{Y73A} under these conditions as compared with earlier analyses in which apo-α_{24}^{Y73A} had been separately incubated with either Isu1_His or Nfs1-Isd11_His.

In striking contrast, upon incubation with holo-α, significant levels of Nfs1-Isd11_His and the bulk of Isu1_His were shifted to higher molecular weight fractions and were co-eluted with newly assembled oligomeric Yfh1 (Fig. 5C, fractions 38–54). A more prominent shift was observed with holo-α_{24}^{Y73A} (Fig. 5E, fractions 38–54) owing to the larger proportion of 24-mer present in this protein preparation. Direct addition of Ni-NTA-agarose beads to fractions eluted from the column enabled the simultaneous pulldown of Yfh1 along with Isu1_His and Nfs1 as well as tagged and untagged Isd11 (supplemental Fig. S5, a and b). Together, these results indicate that simultaneous binding of Yfh1 to Nfs1-Isd11 and Isu1 requires oligomerization of Yfh1 and is stabilized by Fe^{3+} loading of Yfh1 oligomers.

Sulfur Delivery to Isu1 Regulates Iron Release from Oligomeric Yfh1—Binding of Isu1 to Fe^{3+}-loaded oligomeric Yfh1 was not sufficient to induce iron release from Yfh1 (Fig. 3 and not
Core Machinery for Mitochondrial Fe-S Cluster Synthesis

![Oligomerization-dependent interactions of Yfh1 with Nfs1-Isd11His analyzed by pulldown assay](image)

**FIGURE 4.** Oligomerization-dependent interactions of Yfh1 with Nfs1-Isd11His analyzed by pulldown assay. A, assays were performed with 4 μM apo- or holo-Yfh1 proteins without (−) or with increasing concentrations of Nfs1-Isd11His as in Fig. 1 except that 50 μM pyridoxal phosphate was added to the binding buffer. Ni-NTA-agarose-bound protein (100%) was separated on 18% SDS-PAGE and detected by protein staining. S, 2.5 μg (180 pmol) of Yfh1, 8, the amounts of bound Yfh1 pulled down in three independent experiments were measured as in Fig. 18. C, pulldown assays were performed under the experimental conditions described above except that isolated Isd11His was used instead of Nfs1-Isd11His. D, pulldown assays were performed with increasing concentrations of Nfs1-Isd11His incubated with holo-α or untagged Isu1 or both proteins together (4 μM each). Solution conditions were as in A except that 50 mM imidazole and 200 mM NaCl were used to minimize nonspecific binding of untagged Isu1 to Ni-NTA beads. S, 5 μg of Yfh1; S’, 5 μg of Isu1.

shown). We tested whether the ferric iron stored inside oligomeric Yfh1 might be released in the presence of Isu1, elemental sulfur provided as Na2S, and L-cysteine, which is thought to serve as a physiological source of reducing equivalents for reduction of Fe3+ (10, 22). Apo-α and apo-α24Y73A were once again loaded with Fe3+ aerobically to induce oligomerization of wild-type Yfh1 and promote conversion of Fe2+ to Fe3+ inside Yfh1 oligomers. We then used an anaerobic assay monitoring [2Fe-2S] cluster formation by UV-visible absorption analysis over time (10) (Fig. 6A). Reactions containing Isu1His and ferric iron without Yfh1 showed a small increase in A426, relative to control reactions, consistent with some spontaneous assembly of [2Fe-2S] clusters on Isu1His. There was a much greater and faster increase in A426 when holo-α or holo-α24Y73A was incubated together with Isu1His, consistent with Yfh1-catalyzed synthesis of [2Fe-2S] clusters (Fig. 6A). When L-cysteine was not included, the reaction proceeded very slowly, and the yield of [2Fe-2S] clusters after 120 min was only slightly higher compared with a reaction containing Yfh1-bound Fe3+ but no Isu1 (A426 = 0.15 versus 0.11) (not shown).

A lower yield was obtained with holo-α24Y73A. This can be explained by the low iron/subunit ratio (2:1) used in these experiments, which was largely below the maximal iron loading capacity of ~50 iron/subunit. In addition, under these conditions wild-type Yfh1 underwent limited oligomerization, as expected (21), such that the holo-α preparation used in these assays contained ~5 times less 24-mer than the holo-α24 preparation (not shown). Because both preparations contained equal concentrations of protein-bound iron (~47 μM) and Isu1 (6.25 μM), the Fe/24-mer and the Isu1/24-mer ratios were ~5 times lower in the holo-α24 preparation, causing a lower rate of [2Fe-2S] synthesis.

To limit sulfur availability, we replaced 2.5 mM Na2S with low concentrations of Nfs1-Isd11His (0.5–1.0 μM), to provide elemental sulfur via cysteine desulfuration (15). Once again we observed a significant increase in A426 in reactions containing both Yfh1 (provided as holo-α) and Isu1His, which was not observed in control reactions lacking either protein (Fig. 6B). A low yield was obtained with holo-α24Y73A (not shown), as expected given the low Fe/24-mer, Isu1/24-mer, and Nfs1-

 bonded together with Isu1His, consistent with Yfh1-catalyzed synthesis of [2Fe-2S] clusters (Fig. 6A). When L-cysteine was not included, the reaction proceeded very slowly, and the yield of [2Fe-2S] clusters after 120 min was only slightly higher compared with a reaction containing Yfh1-bound Fe3+ but no Isu1 (A426 = 0.15 versus 0.11) (not shown).
Conversely, under anaerobic conditions in which CyaY was stable as Fe$_{2+}$/H$_{1001}$-loaded monomer, the protein inhibited ISC assembly (24). We therefore measured [2Fe-2S] synthesis under anaerobic conditions in which Yfh1 is also known to be stable as Fe$_{2+}$/H$_{1001}$-loaded monomer (25). The rate of [2Fe-2S] synthesis was only slightly increased in the presence of Yfh1 as compared with a reaction in which Fe$_{2+}$/H$_{1001}$ was provided directly (Fig. 6C) and was significantly lower than the rate observed in the presence of Fe$_{3+}$/H$_{9251}$-loaded holo-$\alpha$ (Fig. 6C, inset). These data suggest that anaerobic conditions inhibit the iron-chaperone function of both Yfh1 and CyaY, likely because they prevent Fe$_{2+}$ oxidation and protein oligomerization.

Limited Amounts of Yfh1/Isu1/Nfs1 Complexes are Detected in Vivo—Mitochondrial lysates were prepared from yeast cultures that had been grown without or with iron supplementation under established conditions that cause a small increment in mitochondrial iron content, from $\sim$3 to $\sim$15 nmol/mg of protein, which is associated with limited assembly of endogenous Yfh1 (17). Recombinant Isu1His interacted with significantly larger levels of endogenous Yfh1 or Nfs1 when pulldown assays were performed with mitochondrial lysate prepared from cells supplemented with iron (Fig. 7A). This result is consistent with the idea that Isu1 interacts preferentially with oligomeric Yfh1. The iron-dependent increment in the amount of Nfs1 pulled down by Isu1His could be explained by the presence of a fraction of Nfs1 bound to oligomeric Yfh1 that was pulled down through Isu1His-Yfh1 contacts. Accordingly, Nfs1-Isd11His interacted with significantly larger levels of endogenous Yfh1 in mitochondrial lysate from cells supplemented with iron, although 5-fold higher levels of Yfh1 were required to detect this interaction (Fig. 7, B versus A, top).

In yeast, Yfh1-Y73A exists as a stable trimer that oligomerizes into 24-mer in an iron-dependent manner (17). Accordingly, recombinant Isu1His pulled down endogenous Yfh1-Y73A in mitochondrial lysate from cells supplemented with iron, with an efficiency comparable to that observed with endogenous wild-type Yfh1 (Fig. 7C). In contrast, limited interactions were observed with two Yfh1 proteins lacking ferroxidase activity (Yfh1-D79A;D82A) or iron mineralization activity (Yfh1-E93A;D97A;E103A), which are required for efficient iron-dependent oligomerization of Yfh1 (20). Low rates of iron-dependent oligomerization correlated with limited binding to Isu1His in vitro (supplemental Fig. S6, a–e), whereas there was no detectable binding to Isu1His in mitochondrial lysate (Fig. 7C), probably because the levels of endogenous Yfh1 oligomer interacting with Isu1His were below the limit of detection of our assay.

**DISCUSSION**

In this study we set out to assess how known properties of Yfh1, *i.e.* the ability to bind and oxidize Fe$_{2+}$ and the ability to
Oligomerize, might influence the interactions of Yfh1 with its ISC assembly partners, Isu1 and Nfs1-Isd11. We studied wild-type Yfh1 and the Yfh1-Y73A variant, which oligomerize in an iron-dependent and an iron-independent manner, respectively (17). The Y73A mutation is adjacent to a patch of acidic residues involved in iron binding and oxidation (20, 25), and most likely stabilizes a conformation required to initiate oligomerization (11). In the case of wild-type Yfh1, this conformation is stabilized upon the binding and oxidation of Fe$^{2+}$ (8). Thus, the Y73A mutation enabled us to uncouple oligomerization from iron binding/oxidation, which cannot be independently evaluated in wild-type Yfh1.

Our data show that Yfh1 oligomerization plays an important role in Yfh1-Isu1 interactions as well as Yfh1-Nfs1-Isd11 interactions. The large protein surface of Yfh1 oligomers (18) may facilitate simultaneous contacts with Nfs1-Isd11 and Isu1. Moreover, concurrent binding of oligomeric Yfh1 to both Isu1 and Nfs1-Isd11, and of Isu1 to both Yfh1 and Nfs1-Isd11, may help in stabilizing these multicomponent assemblies. The additional stabilizing effect of the iron stored inside oligomeric Yfh1 may involve iron-mediated contacts between Yfh1 and Isu1 and/or protein conformational changes. Finally, the capacity of Yfh1 oligomers to store iron in ferric form (16, 18) may further provide a mechanism to adjust the rate of iron delivery to that of sulfur delivery, as proposed for prokaryotic ISC assembly (10, 23).

Yfh1 oligomerization is normally tightly coupled with the Yfh1-catalyzed oxidation of Fe$^{2+}$ that leads to formation of a stable ferric mineral within Yfh1 oligomers (5, 16, 21). Previously, this led us to propose that the main function of Yfh1 oligomerization is to detoxify redox-active iron thereby providing mitochondria with antioxidant protection and iron storage capacity (18, 20). The data presented here indicate that Fe$^{3+}$-loaded oligomeric Yfh1 may also function as the iron donor for yeast ISC assembly. Similarly, Fe$^{3+}$-loaded oligomeric CyaY was previously shown to serve as the iron donor for ISC assembly on the IscU scaffold in the presence of the sulfur donor, IscS (10).

Other studies have analyzed iron delivery by Yfh1 or CyaY under strictly anaerobic conditions that inhibit Fe$^{2+}$ oxidation and Yfh1/CyaY oligomerization (24, 26). The physiological relevance of these conditions is uncertain, however, given that both Saccharomyces cerevisiae and E. coli are facultative, not obligate, anaerobes. During aerobic growth these unicellular organisms are directly exposed to atmospheric air, which is expected to result in relatively high intracellular or intramitochondrial oxygen concentrations (27). Notably, under strictly anaerobic conditions, Fe$^{3+}$-loaded CyaY monomer inhibited...
ISC assembly (24), which led to the proposal that CyaY and other frataxin orthologues participate in ISC assembly not as iron chaperones but as iron-dependent inhibitors of ISC formation (24). However, we have shown here that when Fe$^{2+}$ binding to Yfh1 is allowed to occur physiologically under aerobic conditions, the resulting Fe$^{3+}$-loaded oligomers promote ISC assembly on Isu1 (Fig. 6). In contrast, this process is inhibited under strictly anaerobic conditions in which Yfh1 is stable as an Fe$^{3+}$-loaded monomer (Fig. 6).

Although it is possible that changes in oligomeric state enable CyaY and Yfh1 to either promote or inhibit ISC biosynthesis, the available evidence indicates that both CyaY and Yfh1 promote ISC assembly under physiological conditions in vivo (1, 28). Likewise, lack of human or mouse frataxin causes early defects in Fe-S enzymes, prior to, or in the absence of, detectable mitochondrial iron accumulation (29, 30). These data do not support the proposal that frataxin is inhibitory of ISC synthesis and that the primary consequence of frataxin deficiency is an ISC surplus (24). The latter effect would promote iron accumulation and delay appearance of Fe-S enzyme deficiencies, a sequence reverse to that actually observed in vivo (29, 30). It is more likely that the complex phenotypes associated with Yfh1/frataxin deficiency (4) reflect the combined loss of the protein’s iron chaperone and iron storage functions (6, 20).

Because [2Fe-2S] clusters assembled on Isu1 are oxygen-labile (31), anaerobic conditions were needed to measure the ability of Fe$^{3+}$-loaded oligomeric Yfh1 to serve as the iron donor in vitro. Although Fe$^{2+}$, not Fe$^{3+}$, is ultimately present in Fe-S clusters, both [2Fe-2S] and [4Fe-4S] clusters can be formed in vitro (and presumably in vivo) from Fe$^{3+}$ ion, provided that a source of reducing equivalents is present (32). In our in vitro [2Fe-2S] synthesis assay, Yfh1-bound Fe$^{2+}$ was reduced by cysteine and subsequently maintained in the reduced state by the presence of excess cysteine and dithiothreitol, as described for CyaY-bound Fe$^{2+}$ (10). Ferredoxin and ferredoxin reductase are believed to provide reducing equivalents for ISC assembly in vivo (4). Cysteine has also been proposed to serve as a physiological source of reducing equivalents for reduction of Fe$^{3+}$ (22). In addition, both the cysteine desulfurase, IscS, and the scaffold, IscU, were able to reduce Fe$^{3+}$ to Fe$^{2+}$ for prokaryotic ISC assembly (10, 23). Whether Nfs1-Isd11 and/or Isu1 have this ability remains to be established.

Unlike the Y73A variant, wild-type Yfh1 oligomerized at a rate proportional to the iron concentration as expected (5, 17, 21), yielding iron-loaded oligomers that interacted productively with Isu1 and Nfs1-Isd11 even at low Fe/Yfh1 ratios (Fig. 6). This correlated with the situation in vivo where nanomolar concentrations of mitochondrial iron were associated with relatively efficient rates of ISC synthesis in isolated mitochondria (22). In addition, both the cysteine desulfurase, IscS, and the scaffold, IscU, were able to reduce Fe$^{3+}$ to Fe$^{2+}$ for prokaryotic ISC assembly (10, 23). Whether Nfs1-Isd11 and/or Isu1 have this ability remains to be established.

We propose that reversible iron-dependent oligomerization of Yfh1 represents a mechanism to promote mitochondrial ISC assembly.
synthesis and limit iron toxicity. Oligomerization of Yfh1 responds to increments in mitochondrial iron levels (17), and simultaneous interactions of iron-loaded Yfh1 oligomer with Isu1 and Nfs1-Isd11 may enable productive iron release for ISC assembly and prevent potentially toxic increases in the mitochondrial concentration of free iron. Disassembly of wild-type Yfh1 oligomers upon iron release may subsequently facilitate dissociation of holo-Isu1 from Yfh1 and/or enable new associations between holo-Isu1 and molecular chaperones required for the transfer of ISC from holo-Isu1 to appropriate apoenzymes. Additional studies will be required to elucidate this mechanism biochemically and structurally in vitro and in vivo. These studies will be technically challenging but will be critical to achieve a better understanding of how the ISC assembly process functions in physiological conditions and disease states.

REFERENCES

1. Lill, R., and Mühlenhoff, U. (2008) Annu. Rev. Biochem. 77, 669–700
2. Li, K., Tong, W. H., Hughes, R. M., and Rouault, T. A. (2006) J. Biol. Chem. 281, 12344–12351
3. Adam, A. C., Bornhövd, C., Prokisch, H., Neupert, W., and Hell, K. (2006) EMBO J. 25, 174–183
4. Rouault, T. A., and Tong, W. H. (2008) Trends Genet. 24, 398–407
5. Wang, T., and Craig, E. A. (2008) J. Biol. Chem. 283, 12674–12679
6. Fourny, F., Pastore, A., and Trincal, M. (2007) EMBO Rep. 8, 194–199
7. Gerber, J., Mühlenhoff, U., and Lill, R. (2003) EMBO Rep. 4, 906–911
8. Park, S., Gakh, O., Mooney, S. M., and Isaya, G. (2002) J. Biol. Chem. 277, 38589–38595
9. Shan, Y., Napoli, E., and Cortopassi, G. (2007) Hum. Mol. Genet. 16, 929–941
10. Layer, G., Ollagnier-de Choudens, S., Sanakis, Y., and Fontecave, M. (2006) J. Biol. Chem. 281, 16256–16263
11. Karlberg, T., Schagerlöf, U., Gakh, O., Park, S., Ryde, U., Lindahl, M., Leath, K., Garman, E., Isaya, G., and Al-Karadaghi, S. (2006) Structure 14, 1535–1546
12. Gerber, J., Neumann, K., Prohl, C., Mühlenhoff, U., and Lill, R. (2004) Mol. Cell. Biol. 24, 4848–4857
13. Ramelot, T. A., Cort, J. R., Goldsmith-Fischman, S., Kornhaber, G. J., Xiao, R., Shastry, R., Acton, T. B., Honig, B., Montelione, G. T., and Kennedy, M. A. (2004) J. Mol. Biol. 344, 567–583
14. Claros, M. G., and Vincens, P. (1996) Eur. J. Biochem. 241, 779–786
15. Marelja, Z., Stöcklein, W., Nimtz, M., and Leimkühler, S. (2008) J. Biol. Chem. 283, 25178–25185
16. Nichol, H., Gakh, O., O’Neill, H. A., Pickering, I. J., Isaya, G., and George, G. N. (2003) Biochemistry 42, 5971–5976
17. Gakh, O., Smith, D. Y., and Isaya, G. (2008) J. Biol. Chem. 283, 31500–31510
18. Schagerlöf, U., Elmlund, H., Gakh, O., Nordlund, G., Hebert, H., Lindahl, M., Isaya, G., Al-Karadaghi, S. (2008) Biochemistry 47, 4948–4954
19. Park, S., Gakh, O., O’Neill, H. A., Mangravita, A., Nichol, H., Ferreira, G. C., and Isaya, G. (2003) J. Biol. Chem. 278, 31340–31351
20. Gakh, O., Park, S., Liu, G., Macomber, L., Imlay, J. A., Ferreira, G. C., and Isaya, G. (2006) Hum. Mol. Genet. 15, 467–479
21. Adamec, J., Rusnak, F., Owen, W. G., Naylor, S., Benson, L. M., Gacy, A. M., and Isaya, G. (2000) Am. J. Hum. Genet. 67, 549–562
22. Park, S., and Imlay, J. A. (2003) J. Bacteriol. 185, 1942–1950
23. Smith, A. D., Agar, J. N., Johnson, K. A., Frazzon, J., Amster, I. J., Dean, D. R., and Johnson, M. K. (2001) J. Am. Chem. Soc. 123, 11103–11104
24. Adinolfi, S., Iannuzzi, C., Prisci, F., Pastore, C., Iametti, S., Martin, S. R., Bonomi, F., and Pastore, A. (2009) Nat. Struct. Mol. Biol. 16, 390–396
25. Cook, J. D., Bencze, K. Z., Jankovic, A. D., Crater, A. K., Busch, C. N., Bradley, P. B., Stemmler, A. J., Spaller, M. R., and Stemmler, T. L. (2006) Biochemistry 45, 7767–7777
26. He, Y., Alam, S. L., Proteasa, S. V., Zhang, Y., Lesuisse, E., Dancis, A., and Stemmler, T. L. (2004) Biochemistry 43, 16254–16262
27. Turrens, J. F. (2003) J. Physiol. 552, 335–344
28. Vivas, E., Skovran, E., and Downs, D. M. (2006) J. Bacteriol. 188, 1175–1179
29. Puccio, H., Simon, D., Cossée, M., Criquei-Filipe, P., Tiziano, F., Melki, J., Hindelang, C., Matyas, R., Rustin, P., and Koenig, M. (2001) Nat. Genet. 27, 181–186
30. Stehling, O., Elsässer, H. P., Brückel, B., Mühlenhoff, U., and Lill, R. (2004) Hum. Mol. Genet. 13, 3007–3015
31. Raulfs, E. C., O’Carroll, I. P., Dos Santos, P. C., Unciuleac, M. C., and Dean, D. R. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 8591–8596
32. Johnson, D. C., Agar, J. N., Johnson, K. A., Frazzon, J., Amster, I. J., Dean, D. R., and Johnson, M. K. (2001) J. Am. Chem. Soc. 123, 11103–11104
33. Aloria, K., Schilke, B., Andrew, A., and Craig, E. A. (2004) EMBO Rep. 5, 1096–1101
34. Ramazzotti, A., Vanmansart, V., and Foury, F. (2004) FEBS Lett. 557, 215–220
35. Karthikeyan, G., Santos, J. H., Graziewicz, M. A., Copeland, W. C., Isaya, G., van Houten, B., and Resnick, M. A. (2003) Hum. Mol. Genet. 12, 3331–3342

Core Machinery for Mitochondrial Fe-S Cluster Synthesis