Interaction with Cfd1 Increases the Kinetic Lability of FeS on the Nbp35 Scaffold*

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Background: A Cfd1 and Nbp35 heterocomplex serves as scaffold for cytosolic iron-sulfur cluster assembly.

Results: Deficiency in Cfd1-Nbp35 interaction impaired iron turnover on Nbp35.

Conclusion: Cfd1 promotes binding and transfer of labile iron-sulfur cluster on the Nbp35 scaffold.

Significance: This is the first insight into the unique roles of these P-loop ATPases in cytosolic iron-sulfur cluster assembly.

P-loop NTPases of the ApbC/Nbp35 family are involved in FeS protein maturation in nearly all organisms and are proposed to function as scaffolds for initial FeS cluster assembly. In yeast and animals, Cfd1 and Nbp35 are homologous P-loop NTPases that form a heterotetrameric complex essential for FeS protein maturation through the cytosolic FeS cluster assembly (CIA) pathway. Cfd1 is conserved in animals, fungi, and several archaeal species, but in many organisms, only Nbp35 is present, raising the question of the unique roles played by Cfd1 and Nbp35. To begin to investigate this issue, we examined Cfd1 and Nbp35 function in budding yeast. About half of each protein was detected in a heterocomplex in logarithmically growing yeast. Nbp35 readily bound 55Fe when fed to cells, whereas 55Fe binding by free Cfd1 could not be detected. Rapid 55Fe binding to and release from Nbp35 was impaired by Cfd1 deficiency. A Cfd1 mutation that caused a defect in heterocomplex stability supported iron binding to Nbp35 but impaired iron release. Our results suggest a model in which Cfd1-Nbp35 interaction increases the lability of assembled FeS on the Nbp35 scaffold for transfer to target apo-FeS proteins.

Iron-sulfur (FeS) proteins are involved in a wide variety of cellular functions, several essential to life itself (1). The compartmental nature of the eukaryotic cell coupled with the variety and ubiquitous distribution of iron-sulfur (FeS) proteins necessitates multiple systems to ensure efficient FeS protein maturation throughout the cell. All FeS cluster biogenesis in the eukaryotic cell requires the activity of the mitochondrial iron-sulfur (FeS) protein (ISC) assembly system (2). Although small amounts of some components of the ISC system have been detected in the cytoplasm of animal cells (3–5), most cytosolic and nuclear FeS proteins require the action of a cytosolic iron sulfur cluster assembly (CIA) system for their maturation (1, 6–10). The CIA system is restricted to the cytoplasm and consists of six core proteins, in yeast called Cfd1, Nbp35, Nar1, Cia1, Dre2, and Tah18 (1, 6–11). CIA is linked to the ISC system through a yet undefined product that is exported out of mitochondria via the ISC export system (2, 12).

Cfd1 and Nbp35 are P-loop NTPases that are thought to function as scaffolds for the initial assembly of FeS clusters in the CIA pathway (13, 14). These homologous proteins are members of the ApbC/Nbp35 subfamily of P-loop NTPases that is characterized by a conserved C-terminal ENMS sequence followed by a CX2C cysteine cluster (15, 16). This C-terminal cysteine cluster confers the ability onto these proteins of binding a bridging [4Fe-4S] cluster between monomers and is essential for activity of both proteins (6, 14). Nbp35 also has a cysteine cluster at the N terminus, a feature that distinguishes it from Cfd1 and allows the Nbp35 monomer to bind an additional [4Fe-4S] cluster (7, 14). Cfd1 and Nbp35 form a heterotetrameric complex that upon cluster reconstitution in vitro binds up to four [4Fe-4S] clusters, two clusters bridging monomers, and one cluster coordinated at the N terminus of each Nbp35 monomer (14). It is currently unknown whether the bridging clusters are between a homodimer or heterodimer within the heterotetrameric arrangement.

The FeS clusters that assembled in vitro on the Cfd1-Nbp35 heterotetramer, or on each protein independently, were readily transferred to target proteins, supporting the view that these CIA factors serve as scaffolds for initial FeS cluster assembly (13). The ability to coordinate FeS cluster and donate cluster to apo target proteins is a conserved feature of members of the ApbC/Nbp35 family. Ind1 in mitochondria of mammals (17), ApbC in bacteria and archaea (18, 19), and chloroplast HFC101 (20) and AtNBP35 (21) in plants were each shown to coordinate and transfer reconstituted FeS clusters in vitro. Interestingly, in vitro assembly and transfer of FeS clusters on these P-loop NTPases did not require nucleotide binding or hydrolysis. However, nucleotide binding and hydrolysis are required for iron binding to Cfd1 and Nbp35 in vivo (14).

Members of the ApbC/Nbp35 family are widely distributed, being found in virtually all organisms in the biosphere (17, 20, 22). Intriguingly, the requirement for two such P-loop NTPases within...
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the same pathway for FeS cluster biogenesis to date has only been demonstrated in animals and fungi (23). Cfd1 is absent in plants and bacteria. The fact that Nbp35 can act alone in a wide range of organisms raises the question of the unique role of Cfd1 and the need for two P-loop NTPases for cytosolic FeS cluster assembly in animals and fungi. Here we investigated this question by examining the role of Cfd1 and Nbp35 in budding yeast. Our results suggest a model for Cfd1 function in which its interaction with Nbp35 alters the character of Nbp35-bound FeS, making it more labile and enhancing transfer to apo target FeS proteins.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, Media, and Growth Conditions—The 0615d strain (MATa, ura3-52, trp1-Δ63, his3-Δ200, aco1-1, ade2, IDP249) was the parental strain used throughout this study and is described elsewhere (24). To generate Δcfd1 and Δnbp35 strains, the chromosomal copy of each gene was deleted in merodiploid strains using the one-step gene disruption method (25). Briefly, 0615d was transformed with either CFD1 or NBP35 on a CEN/ARS plasmid carrying a URA3 selectable marker (pRS316 (26)). CFD1 or NBP35 on the chromosome was then deleted by targeted gene disruption using TRP1 (26) for CFD1 (deletion from 289 nucleotides upstream to 406 nucleotides downstream of the translation start codon) or a KanMX cassette (27) for NBP35 (deletion of the entire ORF). Gene disruptions were confirmed by PCR amplification of the corresponding chromosomal locus. To construct strains carrying specific CFD1 or NBP35 mutants, the deletion strains were transformed with the indicated mutant gene on a CEN/ARS plasmid (26) followed by counter selection on medium supplemented with 5-fluoroorotic acid (1 mg/ml), selecting for strains that lose the plasmid carrying the wild-type gene (28). CFD1 and NBP35 mutant genes were constructed by site-directed mutagenesis employing a two-step PCR approach (29). Sequencing was performed at the University of Illinois at Chicago Research Resource Center sequencing facility and was compared with published sequences found in the Saccharomyces Genome Database. Yeasts transformations followed the lithium acetate method (30). Transformed yeast cells were grown at 30 °C in minimal medium supplemented with 2% dextrose (SD (31)) and lacking nutrients as necessary for selection and maintenance of specific plasmids. Yeast were grown to mid log phase (A600 ~0.8) for all labeling and co-immunoprecipitation experiments.

Immunoprecipitation—Proteins of interest were expressed with a C-terminal epitope tag, and immunoprecipitations were performed using antibodies specific to the epitope tag. Epitope-tagged versions of proteins were determined to be functional at a level comparable with untagged proteins by gene complementation analysis. For immunoprecipitation, yeast expressing epitope-tagged proteins were pelleted, washed, and lysed by vigorous agitation with glass beads (0.5 mm) in YBB/EDTA (50 mM Tris-Cl, pH 8, 50 mM KCl, 10% glycerol, and 1 mM EDTA) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). Crude cell lysates were cleared by centrifugation at 16,000 relative centrifugal force for 3 min. Protein concentrations of the cleared cell lysates were determined spectrophotometrically (32) and adjusted to equal protein concentration with YBB/EDTA supplemented with Triton X-100 (0.1% final concentration). The cleared cell extract (1.25 mg of total protein) was then incubated with the indicated monoclonal antibody (Cell Signaling) and protein G agarose beads (Santa Cruz Biotechnology) for 2 h at 4 °C. Agarose beads were collected by centrifugation at 1000 relative centrifugal force for 30 s and washed three times with YBB/EDTA + 0.1% Triton X-100. SDS-PAGE sample buffer was added to the washed beads, and half of each reaction was evaluated for precipitated and co-precipitated protein by Western blot. The relative amount of protein precipitated was determined from images of immunostained filters by densitometry using an Alpha Imager (Alpha Innotech) with AlphEaseFC software. Each protein was immunoprecipitated with greater than 90% efficiency as determined from the protein remaining in the post-immunoprecipitation extracts as compared with untreated extracts.

To investigate the amount of Cfd1 or Nbp35 that was engaged in the heterocomplex, ~80% of the supernatants from the first (primary) immunoprecipitation was subjected to a second round of immunoprecipitation with antibody specific to epitope-tagged Cfd1 or Nbp35 or was mock precipitated with protein G-agarose beads alone. The resulting immunoprecipitates were analyzed for precipitated protein by Western blot or associated 55Fe (see below) by liquid scintillation.

55Fe Labeling and Protein-associated Iron Stability Analysis—Yeast were labeled to steady state with 55Fe by growing cells for four doublings in SD medium containing 55FeCl3 (1 μCi/ml, ~1.2 μM total iron concentration). To determine the stability of protein-bound 55Fe, 100 μM 2’,2’-bipyridyl (BIP; Sigma-Aldrich) was added directly to media, and aliquots were removed for measurement of protein-bound 55Fe at various points after BIP addition. Labeled cells were harvested, washed two times with 1 mM EDTA, washed once with YBB/EDTA, flash-frozen in a dry ice/ethanol bath, and stored at ~80 °C until processed. For analysis of protein-bound 55Fe, cell lysate preparation and immunoprecipitation were performed in an anaerobic glove box using oxygen-free buffers. Immunoprecipitations were performed with 2.5 mg of total extract protein as described above.

For pulse labeling, cells were first resuspended in 0.5× the original volume of SD medium containing 100 μM BIP and allowed to grow for 3 h to deplete available cellular iron and stimulate the high affinity iron transport system (33). Cells were collected, washed two times with H2O at room temperature, and resuspended in 0.1× the original volume of iron-free SD medium (Q.BIOgene) supplemented with 1 μCi/ml 55FeCl3 (~0.1 μM iron) and 1 mM ascorbic acid. Aliquots of cells were harvested at designated time points and added to an equal volume of crushed ice to stop cellular iron uptake. Cells were washed and lysed, and protein-bound 55Fe was evaluated by immunoprecipitation of specific proteins. For pulse-chase experiments, cells were pulse-labeled for 30 min as described above followed by chase with 100 μM Fe(NH4)2(SO4)2, also in the presence of 1 mM ascorbic acid.

To investigate the stability of Nbp35-bound 55Fe in cfd1 mutant strains, yeast were grown overnight in iron-free medium, at which point cells were collected, washed, and resuspended into 0.1× the original volume of iron-free medium supplemented with 1 μCi/ml 55FeCl3 (~1 μM iron) and 1 mM ascorbic acid. Cells were allowed to incorporate 55Fe for 30 min, at which time vehicle or BIP was added as indicated in Fig. 8.
and incubation continued a further 30 min. Nbp35 was immunoprecipitated from cleared cell extracts, and $^{55}$Fe was measured by liquid scintillation.

**Enzyme Assays**—Assays for aconitase activity from mammalian IRP1, Leu1, and protein immunoblots were performed as described elsewhere (6, 24, 34, 35).

**RESULTS**

**Heterocomplex Formation by Cfd1 and Nbp35**—Cfd1 and Nbp35 interact, forming a heterotetrameric complex that binds up to four [4Fe-4S] clusters upon reconstitution in vitro (13, 14). It was of interest to determine the fraction of each protein engaged in such a heterocomplex within the yeast cell. To this end, yeast expressing epitope-tagged Cfd1 (HA) and Nbp35 (Myc) were labeled to steady state with $^{55}$Fe, and cleared extracts were subjected to immunoprecipitation (IP) with protein G-agarose beads alone (Mock) or with epitope-specific antibodies to each protein as indicated. A, left panel, proteins in IP samples were separated by SDS-PAGE and transferred to PVDF membranes, and specific proteins (indicated on the left) were detected by immunoblot (WB). The protein targeted in each primary IP is indicated on the top of the figure. Right panel, proteins remaining in the supernatants after the primary IP (Post-IP Extract) were separated by SDS-PAGE and probed for epitope-tagged Cfd1 or Nbp35 by immunoblot as described above. Relative protein levels were determined by densitometry performed on immunoblot images as described under “Experimental Procedures.” Results shown are representative of three independent experiments. B, approximately 80% of the post-IP extracts were subjected to a second round of precipitation with antibodies specific for Nbp35. $^{55}$Fe in precipitates resulting from both the first and the second IPs was quantified by liquid scintillation counting. Nonspecifically associated radioactivity was determined by performing the second precipitation with protein G-agarose beads alone and was subtracted from that obtained with epitope-specific antibodies. **Primary**, the radioactivity in the first IP. The bars on the right of the graph show the radioactivity in IPs of Nbp35 from extracts that had been immunodepleted with beads alone (Mock) or Cfd1-specific antibodies, as indicated. Inset boxes above each bar show the Nbp35 protein recovered in the second IP. **Secondary**, analysis as described in B but for Cfd1-associated $^{55}$Fe. The bars on the right of the graph show the radioactivity in IPs of Cfd1 from the indicated immunodepleted extracts. nd, no radioactivity was detected above the background. Inset boxes above each bar show the Cfd1 protein recovered in the second IP. Error bars represent the S.D. of three independent experiments.

Iron Distribution between Cfd1, Nbp35, and the Cfd1-Nbp35 Heterocomplex—Cfd1, Nbp35, and the Cfd1-Nbp35 heterotetrameric complex bound one, three, or four FeS clusters, respectively, upon cluster reconstitution in vitro (13, 14). To investigate whether each form of these proteins bound a cluster inside the cell, the supernatants from the primary immunoprecipitation described above were subjected to a second immunoprecipitation with antibody to the other factor, and $^{55}$Fe was measured in the pellets from the primary and secondary immunoprecipitations (Fig. 1, B and C). Significantly (−4×) more radiolabeled iron co-precipitated with Nbp35 as compared with Cfd1 (Fig. 1, B and C). Cfd1 and Nbp35 that were specifically precipitated from extracts depleted of the other protein were taken as the noncomplexed or free form of each protein.

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Precipitation of Nbp35 from extracts that had been depleted of Cfd1 yielded $^{55}$Fe that was 80% of the level obtained from mock-depleted extracts (Fig. 1B). Thus, the major portion of Nbp35-bound $^{55}$Fe (80%) was with free protein, whereas a minor fraction (20%) of the $^{55}$Fe associated with Nbp35 was bound in the Cfd1-Nbp35 heterocomplex at steady state. Precipitation of Cfd1 from Nbp35-depleted extracts yielded no detectable $^{55}$Fe (Fig. 1C), showing that the iron associated with Cfd1 was bound primarily in the Cfd1-Nbp35 heterocomplex. It is important to note that immunoprecipitation of Cfd1 or Nbp35 from mock-depleted extracts yielded $^{55}$Fe at a level comparable with that recovered by primary precipitation (Fig. 1, B and C, compare Primary with Mock). Thus, iron associated with both proteins was stable to the manipulations required for sequential immunoprecipitation and accurately reflected the steady-state distribution of iron among the various forms of these proteins.

$^{55}$Fe Labeling Kinetics Reveal Rapid Iron Binding by Nbp35—An FeS scaffold function predicts that Nbp35 will bind and release iron rapidly. As a first step toward addressing these issues, iron-starved yeast were pulsed with $^{55}$Fe at different total added iron concentrations, radioactive iron accumulated over time in cytoplasmic extracts, and Nbp35 was measured. $^{55}$Fe was added at 0.1 $\mu M$ (close to the $K_m$ of the high affinity iron transport system consisting of Fet3 and Ftr1 (36)), at 1 $\mu M$ (equivalent to the iron concentration in minimal media (31)), and at 10 $\mu M$. At 0.1 $\mu M$ iron, maximum $^{55}$Fe accumulation in cells (Fig. 2, upper panel) and near complete medium iron depletion (not shown) were achieved at the earliest time point taken, 15 min. Cells incubated with 1 $\mu M$ $^{55}$Fe accumulated iron up to 60 min after iron addition (Fig. 2, middle panel), the point where medium iron was exhausted (not shown), whereas at 10 $\mu M$ iron, cells continued to accumulate $^{55}$Fe throughout the 90-min experiment and did not significantly deplete iron in the medium (Fig. 2, lower panel).

Binding of $^{55}$Fe to Nbp35 followed closely the pattern of total iron accumulation in yeast cells. At 0.1 $\mu M$, Nbp35-associated $^{55}$Fe was at maximum by 15 min and remained constant for the remainder of the 90-min experiment (Fig. 2, upper panel). When $^{55}$Fe in the medium was increased to 1 $\mu M$, Nbp35-associated iron rapidly increased to maximum at 30 min and remain relatively stable out to 90 min (Fig. 2, middle panel). At 10 $\mu M$ medium iron, $^{55}$Fe on Nbp35 increased linearly out to 60 min, at which point it appeared to reach a plateau (Fig. 2, lower panel). The observation that $^{55}$Fe on Nbp35 reached a stable plateau even after total $^{55}$Fe accumulation had stopped suggests either that iron on Nbp35 had reached equilibrium with a stable cellular iron pool or that Nbp35-bound iron was very stable.

Stability of Iron Associated with Cfd1 and Nbp35—A scaffold function also predicts rapid iron turnover on Nbp35 and Cfd1. To address this question, the stability of iron associated with Nbp35 was assessed in a pulse-chase experiment. Iron-starved yeast were pulsed for 30 min at 0.1 $\mu M$ $^{55}$Fe followed by a chase with an excess of nonradioactive iron (see “Experimental Procedures”). Upon the addition of nonradioactive iron to these cells, the $^{55}$Fe immuno precipitated with Nbp35 decreased rapidly, dropping 40% by 30 min after initiation of the chase (Fig. 3A). Thus, as predicted of an FeS cluster scaffold, the iron associated with Nbp35 was unstable. By contrast, $^{55}$Fe bound to Nar1 and mammalian IRP1 expressed in yeast was very stable during the chase, in each case showing an initial increase in bound $^{55}$Fe (Fig. 3A). Thus, the iron associated with Nbp35 displayed a much more dynamic character than that associated with target proteins, Nar1 and IRP1.

We also examined the stability of the iron bound to Nbp35 and Cfd1 in yeast labeled to steady state with $^{55}$Fe and then transferred to medium containing the membrane-permeant iron chelator BIP. (Cfd1 was labeled poorly in the short $^{55}$Fe pulse of iron-starved yeast but was sufficiently labeled by the long term steady-state procedure to perform this analysis.) BIP inhibits cellular iron uptake in addition to depleting intracellular labile iron pools, rapidly leading to an iron-starved state and inhibition of iron incorporation into protein (33). Iron bound
transiently or in an unstable manner should be rapidly lost from proteins upon exposure of yeast cells to BIP.

The $^{55}$Fe immunoprecipitated with Cfd1 or Nbp35 decreased rapidly upon exposure of yeast to BIP (Fig. 3B). $^{55}$Fe precipitated with Cfd1 or Nbp35 decreased by $\sim$80 and 40%, respectively, by 15 min after BIP addition. $^{55}$Fe associated with Cfd1 decreased to background level by 30 min, whereas that associated with Nbp35 decreased to background level by 15 min after BIP addition. From 30 to 180 min after BIP addition, $^{55}$Fe associated with Nbp35 showed a much slower rate of decrease. The FeS proteins Nar1 and IRP1 showed a more gradual decrease in $^{55}$Fe concentration. At time 0, BIP (100 $\mu$M) was added, and aliquots were collected at the indicated times. Each protein was immunoprecipitated and assessed for the presence of $^{55}$Fe by liquid scintillation counting. Radioactivity that nonspecifically bound to protein G-agarose beads was determined and subtracted from each sample. Data are presented as a percentage of radioactivity measured relative to the zero time point. Error bars represent the S.D. of three independent experiments.

Identification of a Heterocomplex-defective cfd1 Mutant—Strains bearing viable cfd1 mutant genes were used to gain further insight into the role of Cfd1 in the heterocomplex with Nbp35. The cfd1-I allele carries a mutation at the third nucleotide of the translation initiation codon, changing the AUG to an AUA (6). This mutation causes an $\sim$90% reduction of Cfd1 level as a result of inefficient translation initiation on the mutant mRNA, yielding a strain that is deficient for normal Cfd1. (Note that N-terminally truncated Cfd1 resulting from translational initiation at an in-frame downstream AUG codons is not functional.) A second mutant allele was selected in a screen of a collection of cfd1 point mutants for altered ability to co-immunoprecipitate with Nbp35. A Cfd1 mutant bearing a change of phenylalanine 199 to serine (Cfd1F199S) was detected by immunoblot with anti-HA epitope antibodies. The relative expression of proteins in each strain was determined by Western blot performed on total cell extracts (right panels of A and B).

FIGURE 3. Stability of iron on Cfd1 and Nbp35. A, cells expressing epitope-tagged Nbp35 (■), Nar1 (▲), or IRP1 (▲) were pulse-labeled (30 min) with $^{55}$FeCl$_3$ (1 $\mu$Ci/ml; 0.1 $\mu$M) followed by chase with excess nonradioactive iron ($100$ $\mu$M Fe(NH$_4$)$_2$(SO$_4$)$_3$). Aliquots were removed at the indicated times following the addition of nonradioactive iron, and epitope-tagged proteins were immunoprecipitated as described under “Experimental Procedures.” $^{55}$Fe that co-precipitated with each protein was determined by liquid scintillation counting. Data are shown as the percentage of the radioactivity precipitated at the end of the 30-min pulse (time 0). B, cells expressing epitope-tagged Cfd1 (●), Nbp35 (■), Nar1 (▲), or IRP1 (▲) were labeled to steady state (16 h) with $^{55}$FeCl$_3$ (1 $\mu$Ci/ml; $1.2\mu$M total iron concentration). At time 0, BIP (100 $\mu$M) was added, and aliquots were collected at the indicated times, cell extract was prepared, and each protein was immunoprecipitated and assessed for the presence of $^{55}$Fe by liquid scintillation counting. Radioactivity that nonspecifically bound to protein G-agarose beads was determined and subtracted from each sample. Data are presented as a percentage of radioactivity measured relative to the zero time point. Error bars represent the S.D. of three independent experiments.

FIGURE 4. Effect of Cfd1F199S mutation on P-loop NTPase interactions. Yeast expressing HA-tagged Cfd1 or Cfd1F199S, along with either Myc-tagged Nbp35 (A) or Myc-tagged wild-type Cfd1 (B) were grown to mid-log phase and harvested, and cell extracts were prepared and subjected to immunoprecipitation with Myc epitope-specific antibody, as described under “Experimental Procedures.” Co-immunoprecipitation of HA-tagged Cfd1 or Cfd1F199S was detected by immunoblot with anti-HA epitope antibodies. The relative expression of proteins in each strain was determined by Western blot performed on total cell extracts (right panels of A and B).

FIGURE 5. Affect of Cfd1F199S mutation on P-loop NTPase interactions. Yeast expressing HA-tagged Cfd1 or Cfd1F199S along with either Myc-tagged Nbp35 (A) or Myc-tagged wild-type Cfd1 (B) were grown to mid-log phase and harvested, and cell extracts were prepared and subjected to immunoprecipitation with Myc epitope-specific antibody, as described under “Experimental Procedures.” Co-immunoprecipitation of HA-tagged Cfd1 or Cfd1F199S was detected by immunoblot with anti-HA epitope antibodies. The relative expression of proteins in each strain was determined by Western blot performed on total cell extracts (right panels of A and B).
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**Cfd1 Mutation Affects Iron Binding and Release from Nbp35**—
The results above demonstrate that FeS protein maturation in *cfd1-1* and *cfd1F199S* strains is defective. To gain insight into the mechanism affected by these mutations, the kinetics of iron binding to Nbp35 and downstream FeS protein targets was examined. To this end, yeast were made iron-deficient and transferred to medium containing $^{55}$FeCl$_3$. Aliquots were collected at various times after transfer to $^{55}$Fe-containing medium and analyzed for iron associated with Nbp35, Nar1, or IRP1 (Fig. 7). In cells bearing wild-type Cfd1, $^{55}$Fe rapidly accumulated on Nbp35, reaching a plateau at about 30 min after iron addition (Fig. 7A). The level of radioactive iron associated with Nbp35 remained relatively constant out to 60 min, similar to the results shown in Fig. 2. The Nbp35 labeling kinetics in the *cfd1F199S* strain followed a similar pattern to that seen in a strain with wild-type Cfd1 (Fig. 7A). By contrast, the kinetics of iron binding to Nbp35 in the *cfd1-1* strain was significantly slower and reached only 50% of the level seen with wild-type Cfd1 at 60 min (Fig. 7A).

Iron binding to Nar1 and IRP1 showed a different pattern as compared with Nbp35. First, $^{55}$Fe binding to Nar1 and IRP1 was linear in yeast bearing wild-type Cfd1 out to at least 1 h (Fig. 7, B and C). Second, the effect on $^{55}$Fe binding to these proteins by the two Cfd1 mutations was indistinguishable. Although linear, $^{55}$Fe binding to Nar1 was inhibited by $\sim$50% (Fig. 7B), whereas $^{55}$Fe binding to IRP1 was inhibited by $\geq$80% in both *cfd1* mutant strains (Fig. 7C).

$^{55}$Fe binding to Nbp35 in a yeast strain bearing the Cfd1$^{F199S}$ mutant was near normal, but downstream targets showed significant defects in iron binding. Therefore it was of interest to determine whether the iron associated with Nbp35 had the same character in the Cfd1$^{F199S}$ strain as in the strain with wild-type Cfd1. To this end, the stability of $^{55}$Fe associated with Nbp35 was examined. Yeast were made iron-starved and then transferred to medium containing $^{55}$Fe for 30 min. At this point, vehicle (none) or BIP at 175 or 250 $\mu$M was added, and cultures were incubated for 30 min longer.

The amount of $^{55}$Fe that precipitated with Nbp35 from wild-type Cfd1 cells treated with 175 or 250 $\mu$M BIP was reduced by 26 and 70%, respectively (Fig. 8). Without added BIP, $^{55}$Fe precipitated with Nbp35 increased $\sim$2-fold, indicating that the addition of the chelator inhibited iron binding and promoted iron release from Nbp35. By contrast, the $^{55}$Fe precipitated with Nbp35 from both *cfd1* mutant strains exposed to 175 and 250 $\mu$M BIP remained constant relative to the iron associated with the protein at the end of the 30-min labeling period. It should be noted that BIP did block further binding of iron to Nbp35 in the mutant cells (Fig. 8). Thus, the iron bound to Nbp35 in these *cfd1* mutant strains was much less labile in the presence of BIP. Given that the *cfd1-1* mutation causes severe Cfd1 deficiency, whereas *cfd1F199S* encodes a protein that is defective for heterocomplex formation or stability, these results suggest that Cfd1-Nbp35 heterocomplex formation promotes a more labile state of iron bound to Nbp35.

**Comparative Analysis of Cfd1 and Nbp35 Mutants—** CFD1 and NBP35 are both essential in yeast. However, our data show that yeast can tolerate and survive with severe deficiency in Cfd1 activity. This raises the possibility that Cfd1 provides a catalytic function and that it is more critical that Nbp35 is present in stoichiometric amounts. To explore this possibility, a comparable mutation to that in *cfd1-1* changing the AUG translational start codon for synthesis of Nbp35 to AUA was generated, called *nbp35tsm*. This mutation caused a similar decrease in Nbp35 expression as seen for Cfd1 from *cfd1-1*. Like *cfd1-1* strains, *nbp35tsm* yeast strains were viable, demonstrating that yeast can survive with severely depressed levels of Nbp35 as well. An analysis of indicators of CIA function showed similar defects in FeS cluster biogenesis to that seen for *cfd1-1* yeast (Fig. 9). In particular,
aconitase activity of IRP1 was depressed ~80%, and $^{55}\text{Fe}$ binding to Nar1 was inhibited ~50% in the \textit{nbp35}tsm strain. These findings are consistent with the notion that Cfd1 and Nbp35 function in a heterocomplex where depletion of either protein would reduce the

level of this heterocomplex and cause equivalent decreases in FeS cluster assembly.

To further investigate the equivalence of these CIA factors, we constructed the F251S mutation in \textit{NBP35}. Phe-251 is the equivalent residue to Phe-199 in Cfd1 (Fig. 10A). The F251S mutation in Nbp35 was lethal, in contrast to \textit{cfd1F199S}. Similar to Cfd1F199S, Nbp35F251S was defective for heterocomplex formation/stability and did not co-immunoprecipitate with wild-type Nbp35 (Fig. 11). Moreover, Nbp35F251S failed to restore FeS cluster assembly in a \textit{nbp35}tsm strain as judged from lack of conversion of IRP1 to c-aconitase (Fig. 9). Although these results support the notion that Cfd1 and Nbp35 function in a heterocomplex, they also illustrate the nonequivalence of these factors. Moreover, yeast tolerate deficiency in Cfd1 to a greater degree, suggesting a more fundamental cellular role for Nbp35.

**DISCUSSION**

All known FeS cluster assembly systems are built around a common set of activities, with the initial assembly of a labile FeS cluster on a scaffold protein a key early step (37). Previous work had shown that when co-expressed in \textit{Escherichia coli}, Cfd1 and Nbp35 form a heterotetrameric complex that bound four \[4\text{Fe-4S}\] clusters upon reconstitution \textit{in vitro} (14). Here we report that slightly less than half (40%) of Cfd1 and Nbp35 was
in a complex with each other in logarithmically growing yeast. In yeast grown with $^{55}$Fe, radioactivity was detected with both forms of Nbp35, but only with Cfd1 when bound in the hetero-complex. Significantly, at steady state more $^{55}$Fe was associated with free Nbp35, 80% of total. Moreover, little $^{55}$Fe was detected with Cfd1 in iron-starved yeast given a short pulse of $^{55}$Fe, indicating that nearly all Nbp35-bound $^{55}$Fe in these iron-starved conditions was on free protein. A possible explanation for these results is that Cfd1 binding destabilizes the FeS clusters on Nbp35 to facilitate transfer to target proteins, perhaps making it difficult to capture $^{55}$Fe bound to the Cfd1-Nbp35 complex in iron-starved cells because of a heightened demand for cluster assembly. The rapid decay of $^{55}$Fe from Cfd1 and Nbp35 and the observation that Cfd1 deficiency or mutation (e.g. Cfd1F199S) significantly slowed $^{55}$Fe release from Nbp35 support this view. However, Cfd1 deficiency also reduced $^{55}$Fe binding to Nbp35, suggesting that Cfd1 aids in cluster assembly on Nbp35 as well. These observations are consistent with a model in which the Cfd1-Nbp35 heterocomplex is the platform for assembly of labile FeS clusters for transfer to apo proteins. However, at present we cannot rule out that Cfd1 facilitates assembly of both labile and stable FeS clusters on Nbp35.

FeS occupancy on the Cfd1-Nbp35 scaffold was expected to be dynamic and short lived. Indeed $^{55}$Fe on Cfd1 and Nbp35 turned over relatively quickly, with a half-life significantly shorter than that observed for downstream target FeS proteins Nar1 and mammalian IRP1 (Fig. 3). The half-life of $^{55}$Fe on the latter proteins was on the order of hours, whereas essentially all $^{55}$Fe associated with Cfd1 and half of that with Nbp35 was lost in 30 min or less. Interestingly, $^{55}$Fe precipitated with Nar1 and

**FIGURE 9.** Analysis of FeS cluster assembly in the nbp35$^{tsm}$ mutant strain. A, nbp35$^{tsm}$ yeast were transformed with IRP1 and grown to mid-log phase, and aconitase activity was measured in cleared cell extracts. To look at Nbp35$^{F251S}$ functionality, a nbp35$^{tsm}$ strain was transformed with nbp35$^{F251S}$ in addition to IRP1, and aconitase was measured in cleared cell extracts (nbp35$^{tsm}$ + nbp35$^{F251S}$). B, wild-type or nbp35$^{tsm}$ yeast were transformed with Myc epitope-tagged Nar1. The resulting cells were grown overnight in SD medium containing $^{55}$FeCl$_3$. Cells were harvested and washed, and cleared cell extracts were subjected to immunoprecipitation with Myc-specific antibody. Radioactivity that co-precipitated with Nar1 was detected by liquid scintillation. Results are representative of three independent experiments.

**FIGURE 10.** Sequence comparison of P-loop ATPase CX$_2$C region. A, alignment of the CX$_2$C region primary sequence of Saccharomyces cerevisiae (Sc) Cfd1 and Nbp35 with A. fulgidus (Af) homolog, Af226. Phe-199 of Cfd1 and Phe-251 of Nbp35 are highlighted within the rectangular box along with the homologous residue (Phe-194) in Af226. The CX$_2$C motif that coordinates a bridging FeS cluster between monomers (14) is underlined beneath the Af226 sequence. B, ribbon diagram of the Cx backbone from the crystal structure of the Af226 homodimer (PDB ID: 3KB1). The side-chain aromatic ring of Phe-194 from each monomer is shown as **orange** sticks, revealing the $\pi$ stacking interaction adopted in the crystal structure. The side chains from the CX$_2$C Cys residues are also shown as **yellow** sticks. Note that these residues coordinate a zinc atom in the crystal (not shown).
to associate with clusters when part of a complex with Nbp35. Given the inhibitory effect of Cfd1 deficiency on iron binding to Nbp35, a plausible model then is that Cfd1 interaction with Nbp35 is required for binding of the labile bridging FeS clusters. In support of this view, the C-terminal CX<sub>2</sub>C motif in both proteins is required for function as well as for heterocomplex formation (14). The F199S mutation also destabilized the Cfd1-Nbp35 heterocomplex, but in a short pulse of cfd1<sup>F199S</sup> strains, Nbp35 accumulated <sup>55</sup>Fe similar to that seen for wild-type CFD1 yeast (Fig. 7). Because Cfd1<sup>F199S</sup> did not co-precipitate with Nbp35, this raises the possibility that only Nbp35 directly binds FeS in the heterocomplex, whereas interaction with Cfd1 changes the character of the cluster-coordinating centers on Nbp35, allowing for more efficient assembly and transfer of FeS clusters to target proteins.

In addition to Cfd1, maximum cluster assembly on Nbp35 requires mitochondrial ISC and ISC export systems and the CIA factors Dre2-Tah18 (2, 11). The functions of ISC and Dre2-Tah18 in cytosolic FeS cluster biogenesis have been placed upstream of the Cfd1-Nbp35 interaction in the CIA pathway (11). It is tempting to speculate that mitochondrial ISC, ISC export, and Dre2-Tah18 act together to facilitate assembly of the FeS cluster at the N terminus of Nbp35, whereas Cfd1 is needed for assembly of the C-terminal bridging cluster(s). An intriguing possibility is that assembly of the N-terminal cluster on Nbp35 is a requisite step preceding the assembly of the bridging clusters. However, mutation of either N-terminal or C-terminal cluster ligands impaired the ability of Nbp35 to bind iron in yeast cells, suggesting cooperativity between these sites for cluster assembly and/or stability (14). Further work will be necessary to determine the coordination of FeS cluster assembly at the two sites on Nbp35 and the specific role(s) played by Cfd1 in this process.

A conserved feature among ApbC/Nbp35 protein family members is the presence of a phenylalanine residue (equivalent to Phe-199 in Cfd1) located two residues upstream of the C-terminal bridging cluster(s). An intriguing possibility is that assembly of the N-terminal cluster on Nbp35 is a requisite step preceding the assembly of the bridging clusters. However, mutation of either N-terminal or C-terminal cluster ligands impaired the ability of Nbp35 to bind iron in yeast cells, suggesting cooperativity between these sites for cluster assembly and/or stability (14). Further work will be necessary to determine the coordination of FeS cluster assembly at the two sites on Nbp35 and the specific role(s) played by Cfd1 in this process.

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Deficiency of either Cfd1 or Nbp35 qualitatively and quantitatively resulted in similar defects in FeS cluster biogenesis, consistent with the notion that the functional form of these proteins is a heterocomplex with each other. The fact that both proteins are essential indicates that each contributes a unique function to this heterocomplex. The results presented here demonstrate that Nbp35 binds the major portion of FeS clusters in the heterocomplex, but that Cfd1 enhances this binding and the release of FeS from the heterocomplex. Interestingly, although Cfd1 is found in metazoans, most fungi, and a number of Archaea, many other organisms (e.g. plants) only carry an Nbp35 homolog for cytosolic FeS cluster biogenesis (19, 21, 23). Although it is possible that in the latter organisms the functions expressed in Cfd1 were acquired in a single Nbp35 polypeptide, it is intriguing to speculate that Cfd1 evolved from Nbp35 to provide unique functions important in those organisms that carry it. One such function might be to enhance the ability of the CIA system to compete for limiting pools of cellular iron and sulfur, which then allowed this system to become central to cellular iron sensing in metazoans through IRP1 (16). Further insight into this awaits future studies of these P-loop ATPases and the CIA system.

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