Characterization of Iron-Sulfur Protein Assembly in Isolated Mitochondria

A REQUIREMENT FOR ATP, NADH, AND REDUCED IRON*

Ulrich Mühlenhoff, Nadine Richhardt, Jana Gerber, and Roland Lill‡

From the Institut für Zytobiologie und Zytopathologie der Philipps-Universität Marburg, Robert-Koch Strasse 5, 35033 Marburg, Germany

To study the biochemical requirements for maturation of iron-sulfur (Fe/S) proteins, we have reconstituted the process in vitro using detergent extracts from Saccharomyces cerevisiae mitochondria. Efficient assembly of biotin synthase as a model Fe/S protein required anaerobic conditions, dithiothreitol, cysteine, ATP, and NADH. Cysteine is utilized by the cysteine desulfurase Nfs1p to release sulfan sulfur; ATP presumably reflects the function of the Hsp70 family chaperone Ssq1p; and NADH is used for reduction of the ferredoxin Yah1p involved in Fe/S protein biogenesis. Hence, our assay system faithfully reproduces the in vivo pathway. We have further investigated the involvement of various mitochondrial proteins suspected to participate in Fe/S protein biogenesis. In mitochondrial extracts depleted in Isa1p, Fe/S protein formation was severely decreased. A similar strong decline was observed with extracts from θyh1 mitochondria, indicating that both Isa1p and the yeast frataxin homologue, Yfh1p, are crucial for biogenesis of mitochondrial Fe/S proteins. Conversely, the activities of mitochondrial extracts from Δful1 cells were only moderately reduced, suggesting a dispensable role for Nfu1p. Finally, iron utilization for Fe/S protein formation was imported into the matrix of intact mitochondria in ferrous form in a membrane potential-dependent transport step. Our results represent the first in vitro reconstitution of the entire pathway of Fe/S protein maturation.

Proteins with iron-sulfur (Fe/S) cluster cofactors are ubiquitous in both eukaryotic and prokaryotic organisms (1). They play central roles in various cellular processes that include redox reactions, metabolic catalysis, and the sensing of iron and ambient oxygen levels (2–4). The process of Fe/S protein biogenesis is highly conserved in nature and is mediated by a set of key components that, in bacteria, are frequently encoded by the so-called isc (iron-sulfur cluster assembly) operon (5, 6). Homologues of several of these Isc proteins are found in eukaryotes (7, 8) and in the nif gene cluster of nitrogen-fixing bacteria, where they participate in the formation of the Fe/S cluster cofactors of nitrogenase (9, 10). Other homologues are part of the suf operon, which is thought to be involved in iron and sulfur metabolism in bacteria and intracellular parasites (11, 12). Fe/S protein assembly is initiated by the abstraction of sulfan sulfur from cysteine, a reaction catalyzed by a cysteine desulfurase such as IscS or NifS (13–15). A similar activity of the eukaryotic homologue Nfs1 is likely but hitherto has not been shown. In vitro, cysteine desulfurase is sufficient to mediate the assembly of functional Fe/S proteins from cysteine and ferrous iron. However, in biological systems, the reaction is far more complex and involves proteins such as IscU (yeast Isu proteins) or IscA (yeast Isa proteins) that function as initial iron- and sulfur-binding proteins and as scaffolds for the assembly of a transient Fe/S cluster (16–25). Moreover, specific chaperones such as the Hsp70 and Hsp40 homologues HscA (yeast Ssq1p) and HscB (yeast Jac1p) are involved in vivo (26–31), and the process requires a functional ferredoxin/ferredoxin reductase system, presumably as a source of electrons in an as yet unidentified reduction step (32–34). The reconstitution of the cellular Fe/S cluster formation in vitro has been initiated by pioneering investigations of individual steps of this process in simplified systems that consist of two or at maximum three proteins. These include the investigation of the IscS- or NifS-directed Fe/S cluster formation on IscU and IscA from Escherichia coli and/or Azotobacter vinelandii (16–18, 35, 36), and on NifU from Synechocystis sp. PCC 6803 (37).

In eukaryotes such as S. cerevisiae, Fe/S proteins are most frequently found in mitochondria, although only a few examples have been located in the cytosol and the nucleus (38). Mitochondria play a central role in the biosynthesis of cellular Fe/S proteins in yeast. The ISC assembly machinery of mitochondria is required for the biosynthesis of Fe/S proteins that perform central roles in respiration (i.e. complexes I, II, and III of the respiratory chain) and the citric acid cycle (i.e. aconitase). Most of the components of the ISC assembly machinery are closely related to the bacterial Isc proteins, indicating a bacterial origin for this essential mitochondrial process. Moreover, yeast mitochondria are also involved in the maturation of cytosolic Fe/S proteins such as the isopropyl malate dehydrogenase, Leu1p, or the RNase L inhibitor, Rli1p (8, 21, 33, 34). So far, two mitochondrial proteins with a specific function in the maturation of cytosolic Fe/S proteins have been identified; they are the ABC transporter Atm1p of the inner membrane and the sulfhydryl oxidase Erv1p in the mitochondrial intermembrane space (39). Orthologues of both proteins are found in most eukaryotes, including man, but are absent in bacteria. In addition, glutathione was found to be necessary for maturation of cytosolic, but not mitochondrial, Fe/S proteins.2

* This work was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 286, Fonds der chemischen Industrie, and Deutsches Humangenomprojekt. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 49-6421-286-6449; Fax: 49-6421-286-6414; E-mail: Lill@mailer.uni-marburg.de.

1 The abbreviations used are: Fe/S, iron-sulfur proteins; isc, iron-sulfur cluster assembly; DTT, N,N-dithiothreitol.

2 Sipos, K., Lange, H., Fekete, Z., Ullmann, P., Lill, R., and Kispal, G. (2002) J. Biol. Chem. 277, 26944–26949.
The maturation of Fe/S proteins in *S. cerevisiae* has been studied extensively by a combination of genetic and biochemical techniques that allow the analysis of Fe/S protein formation *in vivo* (reviewed in Ref. 38). The possibility of using intact isolated mitochondria to study Fe/S protein maturation in *in vitro* has recently been shown by demonstrating that mitochondrial ferredoxin is assembled into the holocomplex upon import into isolated mitochondria (41). Although both approaches allow the identification of components involved in Fe/S protein maturation, they are not suitable for mechanistic studies of the particular reactions making up this process. In this study, we have established experimental systems that allow the *in vitro* analysis of the assembly of Fe/S proteins in yeast mitochondria. We report the first *in vitro* reconstitution of the entire pathway of Fe/S protein maturation in mitochondria. Our study has revealed several biochemical requirements of this complex process and provides a basis for further dissection of the molecular mechanism of cellular Fe/S protein formation in eukaryotes.

**MATERIALS AND METHODS**

**Yeast Strains and Cell Growth**—The following strains of *S. cerevisiae* were used: W303-1A, trpl-1, his3, ade2, leu2-3, 112 (which served as wild-type; yfb1 (W303-1A, yfb1::KanR; Ref. 42); yfl1 (W303-1A, yfu1::HIS3, this work); and Gal-IAS1 (21). Cells were grown in rich or minimal media containing the required carbon sources (43). Yeast cells were transformed with plasmid DNA by the lithium acetate method (44).

**Fe Incorporation into Fe/S Cluster Apoproteins in Vitro**—The determination of Fe/S protein formation *in vitro* was carried out using mitochondria isolated from yeast strains overexpressing BIO2 (encoding mitochondrial biotin synthase) under the control of the strong constitutive TDI3 promoter from plasmids p426GPD or p424GPD, respectively (45). Unless stated otherwise, cultures were grown overnight at 30 °C in glucose-containing synthetic minimal media lacking added iron chloride. At an optical density of 1–2 at OD<sub>600</sub> cells were harvested, and mitochondria were isolated as described (46).

*In vitro* experiments were carried out under anaerobic conditions in an anaerobic chamber (COY Laboratories) filled with 90% nitrogen and 10% hydrogen gas. In a standard assay, 50–100 μg of isolated mitochondria from iron-starved cells were resuspended in 250 μl of buffer A (40 mM HEPES, pH 7.4, 50 mM KCl, 1 mM MgSO<sub>4</sub>, 0.6 mM sorbitol) supplemented with 1 mM sodium ascorbate, 1 mM DTT, 10 mM pyridoxalphosphate, and 0.2 mM cysteine. Organelles were lysed with Triton X-100 (0.1% final) and incubated with 5 μCi of <sup>55</sup>FeCl<sub>3</sub> (NEN) for 2.5 to 3 h at 25 °C under anaerobic conditions. The labeling reaction was terminated by the addition of 2 ml EDTA on ice. Membrane debris was removed by centrifugation (10 min at 15,000 × g) and Bio2p was immunoprecipitated from the clarified reaction mixture using antibodies attached to protein A-Sepharose as described previously (8, 48). Radioactivity associated with the precipitated immunobeads was quantified by liquid scintillation counting. The S.E. of the assay was between 5 and 20%. ATP depletion of the reaction mixture was carried out by the addition of 25 mM glucose and 20 units of alcohol dehydrogenase (Sigma) 5 min prior to the addition of radioactive iron. Endogenous NADH was depleted by a 5 min preincubation with 10 mM acetaldehyde and 100 units of alcohol dehydrogenase (Sigma).

**Import of <sup>55</sup>Fe into Isolated Mitochondria in Vitro**—For the analysis of iron import into isolated mitochondria, 100 μg of intact mitochondria prepared from a BIO2-overproducing wild-type strain were incubated with 5 μCi of <sup>55</sup>FeCl<sub>3</sub> for 90 min at 25 °C in 250 μl of buffer A supplemented with 1 mM ascorbate, 1 mM DTT, 2 mM NADH, and 5 mM cysteine. The labeling reaction was terminated by the addition of 2 ml EDTA on ice, and mitochondria were recovered by centrifugation (10 min at 10,000 × g). Mitochondria were lysed in 250 μl of TNETG buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 10% glycerol) for 5 min on ice, membrane debris was removed by centrifugation (10 min at 15,000 × g), and Bio2p was immunoprecipitated from the supernatant as described above.

**Fe/S Protein Assembly Mediated by Recombinant Nfs1p**—For the expression of *S. cerevisiae* NFS1 in E. coli, the coding region was modified by PCR to create an Ndel site and an ATG codon at Ser-68. The PCR product was inserted into the Ndel and BamHI sites of vector pET15b (49). Nfs1p was synthesized in E. coli strain HMS174 (DE3) grown at 30 °C in LB medium supplemented with 50 μM pyridoxalphosphate. Six hours after induction with 1 mM isopropyl-1-thio-b-D-galactopyranoside, cells were harvested, resuspended in TSB buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 75 mM NaCl), and lysed by sonication. The cleared cell extract was fractionated by ammonium sulfate precipitation, and the 35–65% ammonium sulfate fraction was applied to a phenyl-agarose column equilibrated with 15% ammonium sulfate-saturated TSB buffer. Nfs1p was eluted with a gradient from 15 to 60% ammonium sulfate and purified further by gel filtration chromatography on a Sephacryl S300 column in 25 mM Tris-HCl, pH 8.0, 50 mM NaCl. Purified Nfs1p eluted at 100 kDa.

Nfs1p-directed Fe/S protein assembly was carried out using recombinant apoferredoxin from the cyanobacterium *Synechococcus elongatus* as an apo-ferredoxin was prepared from 15 to 60% ammonium sulfate and purified further by gel filtration chromatography on a Sephacryl S300 column in 25 mM Tris-HCl, pH 8.0, 50 mM NaCl. Purified Nfs1p eluted at 100 kDa.

**RESULTS**

**Mitochondrial Detergent Extracts Support the Assembly of Fe/S Proteins**—To investigate the formation of Fe/S proteins in mitochondria *in vitro*, we established a radioassay in which a mitochondrial Fe/S protein in its apoform was labeled with <sup>55</sup>Fe by the presence of detergent lysates of isolated mitochondria. The radiolabeled Fe/S holoprotein was subsequently purified by immunoprecipitation from the reaction mixture, and the amount of radioactive <sup>55</sup>Fe firmly associated with the washed immunobeads was quantified by scintillation counting. As a reporter protein, we chose the mitochondrial Fe/S protein biotin synthase, Bio2p, which could be accumulated at high levels in yeast mitochondria when BIO2 was overexpressed under the control of a strong constitutive promoter. The protein has been routinely used for the *in vivo* measurement of Fe/S protein formation in yeast (for example, see Ref. 39). To obtain mitochondria with a large proportion of Bio2p in its apoform, organelles were prepared from iron-deprived yeast cells cultured in synthetic minimal medium that lacked added iron salts.

A time-dependent formation of Fe/S clusters on Bio2p was observed upon incubation of mitochondrial detergent extracts derived from iron-starved yeast cells with radioactive <sup>55</sup>Fe<sup>2+</sup> at physiological pH (Fig. 1A). No further supplementation was required to obtain this basal activity. The rate of <sup>55</sup>Fe association with Bio2p increased 5-fold under optimized conditions involving supplementation of the mitochondrial extracts with cysteine, NADH, and the reducing agent DTT (see below). This assay under optimized (standard) conditions faithfully reflected the activity of Fe/S clusters on apo-Bio2p because the co-immunoprecipitation of <sup>55</sup>Fe depended on the overexpression of Bio2p and the use of specific antibodies against this protein (Fig. 1B). It also displayed a dependence on cysteine (see below). Furthermore, the rate of <sup>55</sup>Fe incorporation was strongly reduced at lower temperatures, as expected for an enzyme-catalyzed process. The reaction required anaerobic conditions because little Fe/S protein formation was observed in the presence of oxygen (Fig. 1B). Because the immunoprecipitation that followed the initial Fe/S protein-forming incubation was carried out under aerobic conditions, the formation of the Fe/S protein must be the oxygen-sensitive step. The time-dependence of the reaction was linear over the period investigated with a rate of Fe/S protein formation of 0.4 pmol <sup>55</sup>Fe/mg mitochondria/min under optimized conditions (Fig. 1A).

**In Vitro Fe/S Protein Assembly Requires Cysteine and a Dithiol Component**—We used the *in vitro* assay to analyze the biochemical requirements for mitochondrial Fe/S protein formation. At first, we investigated the effect of cysteine be-
cause this is the presumed substrate of the mitochondrial cysteine desulfurase, Nfs1p, which is thought to provide the sulfur moiety for Fe/S clusters in vivo (8, 52). The activity of mitochondrial extracts was increased 3-fold upon supplementation with cysteine (up to 0.2 mM in the presence of 1 mM DTT and 0.2 mM NADH) (Fig. 2A). The dependence of the 55Fe association with Bio2p on the addition of cysteine shows that indeed an Fe/S cluster was formed.

Essentially all in vitro Fe/S cluster reconstitution protocols that involve homologues of Nfs1p to generate the sulfur atoms of the Fe/S cluster from cysteine include compounds with reduced thiol groups such as β-mercaptoethanol or DTT. Addition of DTT largely increased the Fe/S protein formation activity of mitochondrial extracts in vitro (Fig. 2B). The stimulatory effect was concentration-dependent with a maximum at 1 mM DTT. Other thiol group-containing biochemicals such as β-mercaptoethanol and reduced glutathione only slightly stimulated Fe/S protein maturation by mitochondrial extracts, with stimulatory effects of 33 and 15%, respectively, of that achieved by DTT at the same concentration (Fig. 2B). Methionine had no effect, and the presence of the reducing agent dithionite had an adverse influence on Fe/S cluster formation on Bio2p, indicating that the stimulatory effect of DTT was not the result of its reducing capacities but rather involves the specific chemistry of thiol groups.

Cysteine desulfurases efficiently transfer the sulfur atom from cysteine to dithiol group-containing biochemicals such as DTT (13, 14). We therefore tested whether yeast Nfs1p functions as a cysteine desulfurase, and we monitored the effect of the thiol compounds on the Nfs1p-mediated reaction. A bacterial [2Fe-2S]ferredoxin in its apoform was incubated in the presence of purified recombinant Nfs1p with cysteine and radioactive 55Fe under anaerobic conditions. Holoprotein formation was subsequently quantified by determining the radioactive 55Fe incorporation into Bio2p. As shown in Fig. 3, Nfs1p catalyzed the efficient formation of holoferrredoxin, yet the reaction required the presence of sulfhydryl-containing compounds. DTT had an over 100-fold stimulatory effect, whereas β-mercaptoethanol and reduced glutathione supported the process only at 25 and 9%, respectively, of the rate in the presence of DTT. Thus, the stimulatory effects of these sulfhydryl-containing biochemicals on the Nfs1p-directed Fe/S protein formation were strikingly similar to those observed with mitochondrial extracts. In addition, the inclusion of reducing agents that lack sulfhydryl groups, such as NADH or dithionite, had little or no effect (Fig. 3). Taken together, Nfs1p functions as a cysteine desulfurase. A dithiol compound appears to be crucial for efficient assembly of Fe/S proteins in vitro. The physiological counterpart of DTT remains unknown at present.

Energy Requirements of Fe/S Protein Formation—We used the in vitro Fe/S protein assembly system to investigate the energy requirements of Fe/S protein assembly. A dependence on NADH was reasonable to expect, because Fe/S protein formation in vivo involves a ferredoxin that presumably is reduced by NADH in a reaction mediated by ferredoxin reductase (33, 34). Supplementation of the in vitro assay system with up to 0.2 mM NADH resulted in a 1.6-fold increase in Fe/S protein formation (Fig. 4A). Higher concentrations had no further beneficial effect. When mitochondrial extracts were depleted of

FIG. 1. Extracts of yeast mitochondria support the assembly of Fe/S proteins in vitro. A, mitochondria isolated from iron-starved wild-type yeast cells overexpressing mitochondrial Bio2p were lysed in detergent-containing buffer, and Fe/S protein assembly was initiated by the addition of radioactive iron. Incubation was carried out in the absence (■) or presence (▲) of 0.2 mM cysteine, 0.2 mM NADH, and 1 mM DTT at 25 °C under anaerobic conditions for the indicated times. Labeling reactions were terminated with EDTA, Bio2p was immunoprecipitated with α-Bio2p-antibodies, and the amount of radioactive 55Fe associated with the immunobeads was quantified by liquid scintillation counting. B, experiments were carried out for 2.5 h as described in A under standard conditions (with 1 mM DTT, 0.2 mM NADH, and 0.2 mM cysteine). One sample contained mitochondria that did not harbor overexpressed Bio2p (-Bio2p), whereas in another sample the immunoprecipitation was performed with antibodies derived from preimmune serum (PIS). Another two samples were incubated at 0 °C or in the presence of oxygen (aerobic).

FIG. 2. The in vitro assembly of mitochondrial Fe/S proteins requires cysteine and a dithiol compound. A, effect of cysteine concentration on Fe/S protein maturation. Mitochondrial detergent lysates were radiolabeled with 55Fe and varying amounts of l-cysteine at 25 °C under standard conditions in the presence of 1 mM DTT and 0.2 mM NADH. 55Fe incorporation into Bio2p was determined as in Fig. 1. B, effect of sulfhydryl-containing compounds on mitochondrial Fe/S protein formation in vitro. Mitochondrial lysates were incubated as in Fig. 1 with thiol-containing compounds at 1 mM final concentration plus 0.2 mM NADH and 0.2 mM cysteine. GSH, reduced glutathione; Dithionite, Na2S2O4; Met, methionine.
Fe/S Protein Formation in Vitro Requires Components of the ISC Assembly Machinery and Frataxin—We studied the need for components of the ISC assembly machinery for Fe/S protein maturation in vitro. At first we analyzed mitochondrial extracts from the strain Gal-ISA1, which carries the ISA1 gene under the control of a galactose-inducible GAL1–10 promoter (21). This strain shows a specific defect in the maturation of cellular Fe/S proteins in vitro upon depletion of Isa1p by growth in the presence of glucose. In the in vitro assay, mitochondria isolated from Gal-ISA1 cells grown in the presence of galactose showed Fe/S protein formation activities similar to wild-type mitochondria (Fig. 5A). In contrast, organelles from Isa1p-depleted Gal-ISA1 cells elicited only 20% of the wild-type activity. Because the levels of Bio2p were similar in the presence and absence of Isa1p (Fig. 5B), the data demonstrate the crucial involvement of Isa1p in the biogenesis of mitochondrial Fe/S proteins.

endogenous NADH by the addition of alcohol dehydrogenase and acetaldehyde, Fe/S protein formation decreased to levels below those observed in the absence of added NADH. Nevertheless, the residual amount of Fe/S protein maturation occurred at about half the optimal rate, indicating that NADH was either not fully depleted by alcohol dehydrogenase treatment or that it is not absolutely essential for mitochondrial Fe/S protein formation in vitro. Most likely, electrons can also be provided from other sources. In this context, we found that NADPH may be substituted for NADH; NADPH supported the in vitro Fe/S protein formation at almost the same rate as that observed with NADH (Fig. 4A).

We next analyzed the requirement of ATP for Fe/S protein maturation. ATP is presumed to be utilized by the chaperone Ssq1p, which is thought to cooperate with its co-chaperone Jac1p in Fe/S cluster assembly. However, addition of ATP to the reaction mixtures had opposing effects on the rates of Fe/S protein formation in vitro, most likely because ATP is an efficient iron chelator at higher concentrations and thus may reduce the availability of radioactive $^{55}$Fe in the assay (Fig. 4B). Therefore, the involvement of ATP in Fe/S protein maturation was studied by depletion of the endogenous ATP levels present in mitochondrial extracts with hexokinase and glucose treatment. Under these conditions, a 7-fold decline in ATP was either not fully depleted by alcohol dehydrogenase (ADH) plus acetaldehyde (AA) prior to the addition of $^{55}$FeCl$_3$. B, the endogenous ATP pool of mitochondrial extracts was depleted by pre-incubation with hexokinase and glucose (HK). Incubations were carried out in the presence of 0.2 mM cysteine and 1 mM DTT, unless indicated otherwise.

**Fig. 3.** $Nfs1p$ is a cysteine desulfurase and requires sulfhydryl-containing compounds for Fe/S protein maturation. Bacterial apoferredoxin (Fd) was incubated with recombinant purified $Nfs1p$ and $^{55}$Fe in the presence of thiol-containing chemicals at 1 mM final concentration at 25 °C under standard conditions for 90 min. Radiolabelling was terminated with EDTA. Holoferredoxin was re-isolated from the reaction mixture by ion-exchange chromatography. The incorporation of radioactive $^{55}$Fe into ferredoxin was determined by scintillation counting.

**Fig. 4.** The in vitro assembly of mitochondrial Fe/S proteins requires ATP and NADH. Detergent lysates of iron-starved mitochondria were incubated as in Fig. 1 with varying amounts of NADH (A) and ATP (B) at 25 °C with $^{55}$Fe under standard conditions. The amount of Fe/S protein formation was followed by determination of the incorporation of radioactive $^{55}$Fe into Bio2p as in Fig. 1A, the amount of endogenous NADH was depleted by incubation with alcohol dehydrogenase (ADH) plus acetaldehyde (AA) prior to the addition of $^{55}$FeCl$_3$. B, the endogenous ATP pool of mitochondrial extracts was depleted by pre-incubation with hexokinase and glucose (HK). Incubations were carried out in the presence of 0.2 mM cysteine and 1 mM DTT, unless indicated otherwise.

**Fig. 5.** The in vitro assembly of Fe/S proteins in mitochondrial extracts requires components of the ISC assembly machinery and frataxin. A, in vitro Fe/S protein assembly in mitochondrial lysates from wild-type cells and the yeast strains Gal-ISA1, $\Delta$nu1, and $\Delta$yfh1. To allow the expression of ISA1 in Gal-ISA1 cells, mitochondria were isolated from cells overexpressing Bio2p grown in iron-poor minimal medium supplemented with glucose or galactose. Mitochondrial detergent lysates were incubated with $^{55}$Fe at 25 °C under standard conditions, and the amount of $^{55}$Fe assembled into Bio2p was determined by immunoprecipitation as described in Fig. 1. B, immunostain of the Fe/S proteins Bio2p and Aco1p in iron-starved mitochondria from wild-type (WT), Gal-ISA1, $\Delta$nu1, and $\Delta$yfh1 cells.

The assay was further used to assess the role of $Nfu1p$ in Fe/S protein formation. $Nfu1p$ shares limited sequence homology with the C-terminal region of NifU, a protein required for
the maturation of the Fe/S clusters of nitrogenase (20, 53). Deletion of *NFU1* in yeast, however, has not revealed any specific defects in cellular Fe/S protein maturation. In vitro Fe/S protein formation in mitochondrial extracts from Δnfu1 cells exhibited 40% reduced activity compared with wild-type samples. Immunostaining shows wild-type expression levels of Bio2p in mitochondria from Δnfu1 cells (Fig. 5B). Hence, these data suggest that Nfu1p plays a role in Fe/S protein formation, yet the effect of the deficiency in Nfu1p on Fe/S protein formation in vitro was rather small, indicating a dispensable involvement of Nfu1p in this process.

Mammalian frataxin and its yeast frataxin homologue, Yfh1p, play an important role in iron homeostasis of eukaryotic cells (42, 54, 55). An involvement of frataxin in Fe/S protein formation has been suspected. However, this protein is not a component of the bacterial *isc* operons, and its role in Fe/S protein maturation has so far not been analyzed. Using mitochondria isolated from a yeast strain in which the frataxin gene was deleted (Δyfh1 cells), we asked whether Yfh1p is involved in mitochondrial Fe/S protein formation in yeast. A strong, almost 10-fold decrease in Fe/S protein maturation in vitro, compared with the wild-type control, was found for mitochondrial extracts deficient in Yfh1p (Fig. 5A). Because the levels of Bio2p in mitochondria from Δyfh1 were similar to those observed in wild-type mitochondria (Fig. 5B), these data strongly suggest that Yfh1p plays a direct and critical role in mitochondrial Fe/S protein formation.

Iron Used in Mitochondrial Fe/S Protein Assembly Is Imported in Reduced Form and Requires a Membrane Potential for Transport—We were further interested in characterizing the requirements for mitochondrial import of iron utilized for Fe/S protein formation within the matrix. Because iron non-specifically associates with biological membranes (47), we employed the Fe/S cluster formation on Bio2p as a tool to indirectly monitor the iron import into isolated mitochondria. The in organelle assembly of Bio2p is a complex process because it involves the transport of iron across the mitochondrial membranes and the formation of an Fe/S cluster and its insertion into the Bio2p apoprotein. Nevertheless, it was possible to discriminate between some of the requirements of the iron import and those of the other partial reactions of the overall process. First, we analyzed the time course of Bio2p maturation in organelle. Mitochondria harboring overexpressed Bio2p were incubated with radioactive ^55^Fe, re-isolated by centrifugation, and lysed by detergent. Radiolabeled Bio2p was subsequently purified from the clarified extracts by immunoprecipitation and quantitated by scintillation counting as described above. Assembly of Fe/S clusters on Bio2p occurred in a time-dependent manner upon incubation of mitochondria with reduced ^55^Fe in the presence of NADH, cysteine, and ascorbate under anaerobic conditions (Fig. 6A). The detected signal appears specific, because it was observed neither with mitochondria that do not overexpress Bio2p nor with radioactive iron chelated by *ortho*-phenanthroline (Fig. 6B). The time-dependence of Fe/S cluster formation on Bio2p was linear with a rate of 0.09 pmol ^55^Fe/mg mitochondria/min. Interestingly, this corresponds to 25% of the rate of Fe/S protein formation observed in mitochondrial detergent lysates. As the most likely explanation, the import of iron into mitochondria is the rate-limiting step of the overall process. Therefore, import can be followed by measuring Bio2p maturation.

The energy dependence of iron import was analyzed. When NADH was omitted in the incubation mixture, a 2-fold slower rate of Bio2p assembly was observed (Fig. 6C). However, because Fe/S protein maturation within mitochondria involves NADH (see above), this result could not readily be taken to suggest a membrane potential requirement for iron import. We therefore depleted the mitochondrial membrane potential by the uncoupler CCCP or the ionophore valinomycin, which caused a 3- to 4-fold reduction in the rate of Fe/S protein formation (Fig. 6, A and C). The residual activity observed after depletion of a membrane potential was most likely caused by a...
small population of mitochondria (5% or less) that became lyased during the long incubation times of the experiments. Thus, the import of the iron utilized for Fe/S protein formation depends on a mitochondrial membrane potential. We next investigated the requirement of ATP for mitochondrial iron import. Addition of ATP to the import mixtures caused a slight reduction in Fe/S cluster formation on Bio2p, which can most likely be attributed to the iron-chelating capacities of ATP at high concentrations (Fig. 6C and Ref. 47). Because our standard assay was not supplemented with ATP outside the mitochondria, the mitochondrial iron import does not appear to require external ATP. Furthermore, no specific cytosolic factors seem to be required for iron import, because the addition of yeast cytosolic extracts did not significantly enhance the formation of mitochondrial Fe/S proteins in vitro (Fig. 6C).

The standard import assay included iron in its reduced (ferrous) form. To analyze whether ferric iron (Fe\(^{3+}\)) was also transported into mitochondria, the import was carried out aerobically in the absence of ascorbate and NADH in order to keep the radioactive iron oxidized. Under these conditions, almost no Fe/S protein formation was observed (Fig. 6D). This strongly suggests that the iron required for Fe/S protein biosynthesis is imported into mitochondria in reduced form. In addition, Fe/S protein formation was only slightly inhibited by addition of a high excess of other divalent heavy metal ions, such as manganese and zinc. This indicates the specificity of the iron import system in mitochondria (see Ref. 47).

**DISCUSSION**

In this study, we have presented an in vitro reconstitution of Fe/S protein maturation in yeast mitochondria, allowing us to define biochemical requirements of this process. Basal rates of Fe/S protein formation were observed in detergent extracts of mitochondria in the presence of ferrous iron without further supplementation, indicating that isolated mitochondria contain all the ingredients necessary to carry out this task. In vitro Fe/S protein maturation requires the exclusion of oxygen, most likely because of the oxygen sensitivity of the Fe/S cluster assembly intermediates that are formed during this process. In this respect, the reducing environment of the mitochondrial matrix may be the most suitable compartment of the eukaryotic cell to carry out this biosynthetic task.

We have identified several low molecular mass components that stimulate the basal rate of Fe/S protein formation within mitochondria, notably, cysteine, ATP, NADH, and dithiol-containing biochemicals such as DTT. The involvement of these compounds in Fe/S protein assembly can be readily integrated into the framework of current knowledge of this complex process (38). Cysteine is the substrate of the mitochondrial cysteine desulfurase Nfs1p, which according to our results can provide the sulfur moiety of Fe/S clusters. The dependence on cysteine of the in vitro Fe/S protein maturation shows for the first time that this amino acid is the sulfur donor for Fe/S clusters in mitochondria. A requirement for NADH as a source of electrons is consistent with the fact that Fe/S protein assembly in vivo involves the ferredoxin reductase/ferredoxin electron transfer chain which uses NADH as an electron donor (33, 34). To our knowledge, our observation is the first account directly demonstrating the involvement of NADH in Fe/S protein assembly. A requirement for ATP is consistent with the fact that two chaperones are specifically involved in Fe/S protein formation, the Hsp70 homologue, Sec1p, and the Hsp40 homologue, Juc1p (28–31). Their bacterial homologues are encoded within the isc operons of, for instance, *E. coli* and *A. vinelandii*; their function in Fe/S protein assembly as a binding partner of IacU has been amply demonstrated (26, 27). Our data indicate an essential requirement for ATP, because the depletion of ATP by hexokinase results in an almost complete breakdown of Fe/S protein formation of mitochondrial extracts.

Previously, the formation of the Fe/S protein ferredoxin in isolated chloroplasts was found to be dependent on ATP and light (56). The latter could be substituted for NADPH. Apparently, both mitochondria and chloroplasts show the same biochemical need for ATP and NADPH to generate Fe/S proteins. These results suggest the involvement of a functionally similar apparatus in the formation of Fe/S proteins in both organelles. To date, none of the components of the plastid ISC assembly machinery is known.

Fe/S protein maturation supported by mitochondrial detergent extracts or catalyzed by isolated yeast Nfs1p required a dithiol group-containing compound. This requirement seems to be specific because biochemicals with a single thiol group were much less or not at all stimulatory. Reducing agents were not effective, demonstrating that the requirement reflects sulfhydryl rather than redox chemistry. Although most in vitro Fe/S protein reconstitution protocols include at least one reducing agent, the basis of their stimulatory effects on Fe/S protein formation so far has not been analyzed systematically. Based on the effective transfer by Nfs1p-like desulfurases of the sulfur atom of cysteine to DTT (14, 57), it seems reasonable to assume that the dithiol compound may function as the immediate acceptor of the sulfur that is bound on Nfs1p following its abstraction from cysteine. In this view, the dithiol may serve as a mobile carrier of the sulfur atom from Nfs1p to the Fe/S apoprotein. However, a direct transfer of sulfur from IacS to IacU has recently been reported, rendering this possibility unlikely (35, 36). Therefore, the role of the dithiol compound remains to be elucidated. Further, the nature of the mitochondrial counterpart of DTT in vivo is currently unknown. The compound may either be a low-molecular mass dithiol-containing molecule, possibly derived from cysteine or methionine, or an unidentified protein that carries two cysteine residues in close vicinity, such as a thioredoxin or a disulfide isomerase. Because of the broad substrate range of these proteins, their potential involvement in Fe/S protein formation may have been overlooked. In this context, Nfu1p, which is supposed to be involved in Fe/S protein maturation in an unknown reaction, contains a typical CXXC sequence motif at its C terminus. We therefore tested whether recombinant Nfu1p may substitute for DTT in the Nfs1p-directed Fe/S protein assembly assay. However, the isolated protein had no stimulatory effect (not shown).

We successfully employed our novel reconstitution system to investigate the function of several mitochondrial proteins in Fe/S protein maturation. A deficiency in both Isa1p and Yhb1p caused a strong decline in Fe/S protein assembly. For Isa1p, this finding was expected because a function of this protein in Fe/S protein maturation has been described previously (21, 23). However, more than a simple confirmation, the in vitro assay narrows down the reasons for the depletion of Isa1p to defects in Fe/S protein maturation within the mitochondria itself. A role in Fe/S protein formation has been proposed for frataxin as one of several alternatives for the cellular function of this protein in eukaryotes (see Ref. 58). In *S. cerevisiae*, the deletion of the homologous gene *YFH1* frequently results in respiration deficiency, which correlates with low activities of mitochondrial Fe/S proteins, a reversible accumulation of iron within the mitochondria, and occasional loss of mitochondrial DNA (42, 55). These phenotypes are also found in yeast strains with mutations in genes that are directly involved in Fe/S protein biosynthesis, such as *ISA1*, *ISA2*, *NFS1*, and *YAH1*. Here, we have shown that the loss of Yhb1p results in direct defects in mitochondrial Fe/S protein formation in vitro, which strongly...
argues in favor of a specific role for Yfh1 in this process and not in general iron homeostasis or iron transport. In contrast, deletion of NFU1 results in only moderate defects in the formation of mitochondrial Fe/S proteins in vitro. Nfuf1 shares limited sequence homology with the C-terminal region of NifU, a protein that is required for the maturation of the Fe/S cluster of nitrogenase (16). The simultaneous deletion of NFU1 and ISU1 is lethal in S. cerevisiae (20). Nevertheless, our data indicate a dispensable role for Nfuf1 in mitochondrial Fe/S protein maturation in yeast. This result is in perfect accordance with the non-conspicuous phenotype of mutants carrying site-directed cysteine mutations in the Nfuf1-like portion of bacterial NifU (40).

We have employed the reconstitution of mitochondrial Fe/S protein maturation in vitro to characterize the import of iron into isolated mitochondria. The import of iron is four times slower than the assembly of Fe/S proteins in mitochondrial matrix extracts and thus may represent the rate-limiting step of Fe/S protein formation in vivo. Similarly, iron import was found to be rate-limiting for ferrochelatase function in heme biosynthesis (47). Iron import for mitochondrial Fe/S protein formation required reduced (ferrous) iron, a mitochondrial membrane potential, and was minimally inhibited by divalent metals. The transport of iron in its reduced form provides the appropriate substrate for the Isu proteins, because the bacterial homologue IscU has been shown to bind ferrous iron (16, 59). The characteristics of the import utilized in Fe/S protein maturation are strikingly similar to the properties reported previously for the import of iron used in heme biosynthesis (47). This strongly suggests that the maturation of Fe/S proteins in the mitochondrial matrix and the formation of heme catalyzed by ferrochelatase at the inner membrane utilize iron that is imported by similar pathways. These findings fit nicely to a recent study suggesting that at least a fraction of the iron imported in heme and Fe/S protein biogenesis might be imported into mitochondria by the carrier proteins Mrsp3 and Mrsp4 (60).

Taken together, our novel experimental approaches allow the molecular analysis of the formation of Fe/S proteins in isolated mitochondria. The ability to monitor defects in mitochondrial Fe/S protein biogenesis in yeast mutants will facilitate future mechanistic analyses of Fe/S protein maturation in yeast. Moreover, the ability to observe Fe/S protein formation in a simplified system reduces the chances of misinterpretation that are more likely when working with whole cells in vivo. So far, our reconstitution approach is restricted to the analysis of Fe/S protein formation with mitochondrial detergent extracts. The knowledge gained from this experimental system will pave the way to reconstitute the entire process of Fe/S protein maturation with purified components.

Acknowledgment—We thank Dr. F. Foury for kindly providing the yeast strain 3yf1.

REFERENCES

1. Feinert, H., Holm, R. H., and Mann, E. (1997) Science 277, 653–659
2. C hamstring, R. (1992) in Iron-Sulfur Proteins (C hamstring, R., ed) Vol. 38, pp. 281–322. Academic Press, San Diego
3. Johnson, M. K. (1998) Curr. Opin. Chem. Biol. 2, 173–181
4. Feinert, H., and Kiley, P. J. (1999) Curr. Opin. Cell Biol. 11, 152–157
5. Zheng, L., White, R. H., Cash, V. L., and Lill, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10206–10211
6. Kaut, A., Lange, H., Dieckert, K., Kaspar, G., and Lill, R. (2000) J. Biol. Chem. 275, 15965–15961
7. Pelzer, W., Muhlenhoff, U., Dieckert, K., Siegmund, K., Kaspar, G., and Lill, R. (2000) FEBS Lett. 476, 134–139
8. Jensen, L. T., and Cullot, V. C. (2000) Mol. Cell. Biol. 20, 3918–3927
9. Wu, G., Maney, S. S., Hemann, C., Hille, R., Surerus, K. K., and Cowan, J. A. (2002) J. Biol. Inorg. Chem. 7, 526–532
10. Silberg, J. J., and Vicker, L. E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7790–7795
11. Silberg, J. J., Hoff, K. G., Tapley, T. L., and Vicker, L. E. (2000) J. Biol. Chem. 275, 1686–1700
12. Voisine, C., Cheng, Y. C., Ohlson, M., Schiule, B., Hoff, K., Beinert, H., Marszalek, J., and Craig, E. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1483–1488
13. Kato, S., Mihara, K., Kurihara, T., Takahashi, Y., Tokumoto, U., Yoshimura, T., and Esaki, N. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5948–5952
14. Ishio, K., and Nakai, M. (2000) J. Biol. Chem. 275, 22615–22618
15. Muhlenhoff, U., and Lill, R. (2000) Biochim. Biophys. Acta 1499, 370–382
16. Langle, H., Kaspar, G., Kaut, A., and Lill, R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1050–1055
17. Li, J., Saxena, S., Pain, D., and Dancis, A. (2001) J. Biol. Chem. 276, 1503–1509
18. Urchiva, H. D., Silberg, J. H., Hoff, K. G., and Vicker, L. E. (2001) J. Biol. Chem. 276, 44521–44526
19. Kato, S., Mihara, K., Kurihara, T., Takahashi, Y., Tokumoto, U., Yoshimura, T., and Esaki, N. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5948–5952
20. Nishio, K., and Nakai, M. (2000) J. Biol. Chem. 275, 22615–22618
21. Muhlenhoff, U., and Lill, R. (2000) Biochim. Biophys. Acta 1499, 370–382
22. Langle, H., Lisozyew, T., Gerber, J., Muhlenhoff, U., Kaspar, G., and Lill, R. (2001) EMBO Rep. 2, 715–720
23. Agar, J. N., Yuvaniyama, P., Jack, R. F., Cash, V. L., Smith, A. D., Dean, D. R., and Johnson, M. K. (2001) J. Biol. Chem. 276, 15022–15027
24. Harlow, E., and Lane, D. (1986) Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorf, J. W. (1990) Methods Enzymol. 185, 60–89
26. Floss, B., Igloi, G. L., Cassier-Chauvat, C., and Muhlenhoff, U. (1997) Photosynth. Res. 54, 65–71
27. Meyer, J., Moulis, J. M., and Lutz, M. (1986) Biochim. Biophys. Acta 871, 243–249
28. Li, J., Kogan, M., Knight, S. A., Pain, D., and Dancis, A. (1999) J. Biol. Chem. 274, 33025–33034
29. Fu, W., Jack, R. F., Morgan, T. V., Dear, D. R., and Johnson, M. K. (1994) Biochemistry 33, 13455–13463
30. Campuzano, V., Montermini, L., Molto, M. D., Planse, L., Cossee, M., Cavalcanti, F., Monros, E., Rodius, F., Duclos, F., Monticelli, A., Zara, F., Canizares, J., Koutnikova, H., Bidichandani, S., Geliera, C., Brice, A., Trouillas, P., DeMichele, G., Filla, A., deFrutos, R., Pialau, F., Patel, F. I., Dihato, S., Mandel, J. A., Cozzone, S., Keog, M., and Pandolfo, M. (1996) Science 271, 1423–1427
31. Babcock, M., De Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Dandolfo, M., and Kaplan, J. (1997) Science 276, 1709–1712
32. Takahashi, Y., Mituji, A., Fujita, Y., and Matsubara, H. (1991) Plant Physiol. 95, 104–110
33. Jakobczyk, K., and Seidler, A. (2000) Biochemistry 39, 3416–3423
34. Puccio, H., and Koenig, M. (2000) Hum. Mol. Genet. 9, 887–892
35. Agar, J. N., Zheng, L., Cash, V. L., Dear, D. R., and Johnson, M. K. (2000) J. Am. Chem. Soc. 122, 2136–2137
36. Foury, F., and Roganti, T. (2002) J. Biol. Chem. 277, 24475–24483