Iron-Sulfur Protein Biogenesis in Eukaryotes: Components and Mechanisms

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cysteine desulfurase, ferredoxin, chaperones, ABC proteins, P-loop ATPase

Abstract
Iron-sulfur (Fe/S) clusters require a complex set of proteins to become assembled and incorporated into apoproteins in a living cell. Researchers have described three distinct assembly systems in eukaryotes that are involved in the maturation of cellular Fe/S proteins. Mitochondria are central for biogenesis. They contain the ISC—the iron-sulfur cluster assembly machinery that was inherited from a similar system of eubacteria in evolution and is involved in biogenesis of all cellular Fe/S proteins. The basic principle of mitochondrial (and bacterial) Fe/S protein maturation is the synthesis of the Fe/S cluster on a scaffold protein before the cluster is transferred to apoproteins. Biogenesis of cytosolic and nuclear Fe/S proteins is facilitated by the cytosolic iron-sulfur protein assembly (CIA) apparatus. This process requires the participation of mitochondria that export a still unknown component via the ISC export machinery, including an ABC transporter.
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## INTRODUCTION

Proteins frequently depend on cofactors to become functionally active as enzymes, regulators, and transporters. Many of these cofactors are chemically related to vitamins such as biotin, panthotenic acid, retinol, or thiamine. Because mammalian cells usually cannot produce such cofactors by their own biosynthetic machinery, they have developed strategies to take up these compounds from the environment, e.g., from bacteria in the gut or from nutrition. Important and ancient cofactors of proteins are the so-called iron-sulfur (Fe/S) clusters. In contrast to most other bio-organic cofactors, these compounds are of inorganic nature and consist simply of iron cations ($\text{Fe}^{2+}$ or $\text{Fe}^{3+}$) and sulfide anions ($\text{S}^{2-}$). Fe/S clusters are inherently labile in free form, and therefore each organism has to assemble them inside the cell de novo. As a prosthetic group of proteins, these structures were discovered and characterized by spectroscopic and analytical chemistry methods in the early 1960s (Beinert et al. 1997, 2004; Rees & Howard 2003). To date, numerous Fe/S proteins are known in each of the three kingdoms of life, i.e., in Eubacteria, Archaea, and Eukarya. These proteins perform important functions in many metabolic reactions, are involved in electron transport, and play a role in gene expression regulation as sensors for environmental or intracellular conditions. The most prominent Fe/S proteins include complexes I, II, and III of the bacterial and mitochondrial respiratory chains; photosystem I and ferredoxin of photosynthesis; nitrogenase of azototrophic bacteria; aconitase of the Krebs cycle; and iron regulatory protein 1 (IRP1) involved in iron-uptake regulation in mammals (Balk & Lobreaux 2005, Lill & Mühlenhoff 2005).

The simplest form of an Fe/S center is the rhombic [2Fe-2S] cluster. A duplication yields the rather common [4Fe-4S] cluster, which possesses a distorted cubic structure with Fe and S atoms at alternating corners of the cube. More complex structures with two or more of these simple Fe/S clusters that are usually
chemically linked are known and well characterized (for detailed review, see Rees 2002, Beinert et al. 2004). In some of these complex Fe/S proteins, other heavy metals such as molybdenum, vanadium, or nickel may replace one of the iron ions or may be present in addition to the iron ions. The binding of the Fe/S clusters to the polypeptide chain occurs through the coordination of the positively charged iron ions by electron donors provided by various amino acid side chains. The most typical and widely used example is the sulfur of cysteine residues. However, the nitrogen of histidine residues or other electron donor groups also may be utilized for cluster binding. Although Fe/S clusters can be assembled efficiently in vitro on numerous purified apoproteins from ferrous iron (Fe$^{2+}$) and sulfide by using chemical reconstitution methods under anaerobic conditions, biosynthesis in a living cell does not occur spontaneously and requires surprisingly complex biochemical assembly systems. The need for assisted rather than spontaneous Fe/S cluster assembly and insertion into apoproteins may mainly be the result of the anaerobic conditions and the toxicity of the high concentrations of iron and sulfide ions required for efficient chemical reconstitution.

This review briefly summarizes the current understanding of the biogenesis of eukaryotic (nonphotosynthetic) Fe/S proteins. Eukaryotes contain Fe/S proteins in mitochondria, the cytosol, and the nucleus (Figure 1).
Because the biogenesis system for mitochondrial Fe/S proteins has been inherited from eubacteria in evolution and consequently shows striking similarities to the bacterial assembly pathways, we frequently refer to seminal findings obtained from work on bacterial proteins. For a more comprehensive summary of the complex and diverse pathways of bacterial Fe/S protein assembly, we direct the reader to recent excellent reviews (Mansy & Cowan 2004, Barras et al. 2005, Fontecave et al. 2005, Johnson et al. 2005). Other accounts on this process in yeast, mammals, or plants have been published previously elsewhere (Balk & Lill 2004, Balk & Lobreaux 2005, Kessler & Papenbrock 2005, Lill & Mühlhoff 2005, Rouault & Tong 2005, Lill et al. 2006). We also briefly discuss the intimate connections of Fe/S protein assembly to iron homeostasis and heme biosynthesis.

SHARED AND DISTINCT Fe/S PROTEIN ASSEMBLY SYSTEMS IN EUKARYOTES

Bacteria have developed (at least) three distinct, highly conserved biosynthetic systems that are involved in Fe/S protein formation. The nitrogen fixation system, NIF, was discovered first and is dedicated to the assembly of the Fe/S clusters of nitrogenase, the enzyme responsible for the conversion of N₂ to NH₃ in nitrogen-fixing bacteria (for recent reviews, see Rees & Howard 2000, Frazzon & Dean 2002, Dos Santos et al. 2004). Key components of the NIF system are the cysteine desulfurase NifS (Zheng et al. 1993) and the scaffold protein NifU (Yuvaniyama et al. 2000) on which the Fe/S clusters are preassembled before insertion into the nitrogenase (Table 1). Other components of the NIF system perform more specific functions for the assembly of the complex clusters of nitrogenase.

The second system, designated ISC (iron-sulfur cluster), is required for the generation of the majority of cellular Fe/S proteins and thus performs a general housekeeping biosynthetic function in bacteria (Figure 1) (Zheng et al. 1998, Takahashi & Nakamura 1999). The two central proteins of the ISC machinery are IscS and IscU, and they are homologous in function to NifS and NifU, respectively (Table 1). These two ISC proteins are encoded by the isc operon, which contains additional genes involved in biogenesis. Strikingly, homologs for all isc operon–encoded components are present in the genomes of eukaryotes (Table 1) (Strain et al. 1998, Kispal et al. 1999, Schilke et al. 1999, Lill & Kispal 2000). The eukaryotic ISC components are located (mainly) in mitochondria and together with some additional proteins are designated as the ISC assembly machinery. Conceivably, this system was inherited from the bacterial endosymbiotic ancestor of this organelle in evolution (Figure 1).

The third bacterial assembly system, termed SUF (sulfur mobilization), plays a similar general role as the ISC system but operates mostly under stress conditions such as iron-limitation or oxidative stress (Figure 1) (for a review, see Fontecave et al. 2005). The SUF system also contains a cysteine desulfurase (SufS) as the sulfur donor and an alternative scaffold protein termed SufA. Sequence homologs of this system are present in plastids of plants and algae, where they perform crucial roles in the biogenesis of Fe/S proteins of the photosynthetic apparatus (Balk & Lobreaux 2005). These organisms may also use Nfu1-like proteins, which are not part of the suf operon, as central scaffolds (see below). Presumably, the SUF system was inherited from the cyanobacterial ancestor of plastids (Figure 1, left side) (Takahashi & Tokumoto 2002).

Whereas organelar Fe/S protein assembly apparently has its evolutionary origin in the biosynthetic systems of bacteria, maturation of Fe/S proteins in the cytosol and nucleus is accomplished by the so-called cytosolic iron-sulfur protein assembly (CIA) machinery that is unique for eukaryotes. CIA components include two P-loop NTPases and an Fe/S
Table 1  Known components of Fe/S protein biogenesis in yeast

<table>
<thead>
<tr>
<th>Name</th>
<th>Essential in yeast</th>
<th>Bacterial homologs</th>
<th>Function</th>
<th>Bound cofactor</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ISC assembly machinery of the mitochondrial matrix</td>
<td></td>
</tr>
<tr>
<td>Nfs1</td>
<td>Yes</td>
<td>NifS, IscS, SufS</td>
<td>Cysteine desulfurase, sulfur donor (Cys → Ala + S), also required for</td>
<td>PLP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thiouridine modification of rRNA</td>
<td></td>
</tr>
<tr>
<td>Isd11</td>
<td>Yes</td>
<td>—</td>
<td>Forms complex with Nfs1, required for sulfur transfer to Isu1</td>
<td>—</td>
</tr>
<tr>
<td>Isu1, Isu2</td>
<td>Yes (double deletion)</td>
<td>NiU (N-terminal domain), IscU</td>
<td>Scaffold for initial cluster assembly, interacts with Nfs1, Yfh1, Ssq1,</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Jac1</td>
<td></td>
</tr>
<tr>
<td>Yah1</td>
<td>Yes</td>
<td>Fdx, middle domain of NiU?</td>
<td>Ferredoxin, reduction of an unknown substrate, possibly S(^0) to S(^2^{-})</td>
<td>[2Fe-2S] cluster</td>
</tr>
<tr>
<td>Arh1</td>
<td>Yes</td>
<td>—</td>
<td>Ferredoxin reductase, electron transfer to Yah1 from NADH</td>
<td>FAD</td>
</tr>
<tr>
<td>Yfh1</td>
<td>No (PET)(^a)</td>
<td>CyaY</td>
<td>Putative iron donor, iron-stimulated binding to Isu1</td>
<td>—</td>
</tr>
<tr>
<td>Ssq1</td>
<td>No, cold sensitivity, strong growth defect</td>
<td>HscA</td>
<td>Specialized Hsp70 chaperone, binds to Isu1, Jac1, transfer of Fe/S clusters to target proteins?</td>
<td>ATP</td>
</tr>
<tr>
<td>Jac1</td>
<td>Yes</td>
<td>HscB</td>
<td>Cochaperone of Ssq1, targets Ssq1 to Isu1</td>
<td>—</td>
</tr>
<tr>
<td>Mge1</td>
<td>Yes</td>
<td>(GrpE, not required in bacteria)</td>
<td>ADP/ATP exchange on Ssq1</td>
<td>—</td>
</tr>
<tr>
<td>Grx5</td>
<td>No</td>
<td>?</td>
<td>Monothiol glutaredoxin</td>
<td></td>
</tr>
<tr>
<td>Isa1, Isa2</td>
<td>No (PET)(^b)</td>
<td>IscA, SufA, IscANif</td>
<td>Biogenesis of aconitase-like Fe/S proteins in yeast, binds iron in yeast and bacteria, functions as alternative scaffold in bacteria</td>
<td>Fe</td>
</tr>
<tr>
<td>Nfu1</td>
<td>No</td>
<td>C-terminal domain of NiU</td>
<td>Unknown function, genetic interaction with Isu1 and Ssq1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mitochondrial ISC export machinery</td>
<td></td>
</tr>
<tr>
<td>Atm1</td>
<td>No (strong growth defect)</td>
<td>Close sequence relative in some bacteria</td>
<td>ABC transporter, inner membrane, export of unknown compound for cytosolic and nuclear Fe/S protein maturation and iron-uptake regulation</td>
<td>ATP</td>
</tr>
<tr>
<td>Erv1</td>
<td>Yes</td>
<td>—</td>
<td>Sulphhydryl oxidase in the intermembrane space, also required for protein import</td>
<td>FAD</td>
</tr>
<tr>
<td>Glutathione (GSH)</td>
<td>Yes</td>
<td>—</td>
<td>Unknown role, redox buffer in yeast</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CIA machinery</td>
<td></td>
</tr>
<tr>
<td>Cfd1</td>
<td>Yes</td>
<td>(ApbC)(^b)</td>
<td>Soluble P-loop NTase, complex with Nbp35, binds labile Fe/S cluster in vitro</td>
<td>ATP or GTP</td>
</tr>
<tr>
<td>Nhp35</td>
<td>Yes</td>
<td>—</td>
<td>Soluble P-loop NTase, binds to Cfd1 and Nar1, can assemble additional, labile Fe/S cluster in vitro</td>
<td>ATP or GTP, Fe/S cluster</td>
</tr>
<tr>
<td>Nar1</td>
<td>Yes</td>
<td>Iron-only hydrogenase</td>
<td>Fe/S protein, binds to Nhp35 and Cia1</td>
<td>2 Fe/S clusters</td>
</tr>
<tr>
<td>Cia1</td>
<td>Yes</td>
<td>—</td>
<td>WD40 domain protein, late function in biogenesis, binds to Nar1, located mainly in nucleus</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\)PET, no growth on glycerol-containing media.

\(^b\)Only two of the four cysteine residues of Cfd1 are conserved in ApbC. See text for further details.
THE BIOSYNTHETIC PRINCIPLE OF Fe/S CLUSTER ASSEMBLY: DESULFURASE PLUS SCAFFOLD PROTEIN

In the NIF, ISC, and SUF systems, the central catalytic step for Fe/S cluster assembly involves a cysteine desulfurase that provides the necessary sulfur ligands and a protein that serves as a molecular scaffold for the initial de novo synthesis of the Fe/S cluster. In eukaryotes such as yeast, the active cysteine desulfurase is a complex of Nfs1 and Isd11, a small protein stabilizing Nfs1 (Figure 2). The eukaryotic scaffold protein is the highly conserved Isu1 (Saccharomyces cerevisiae additionally contains the close homolog Isu2). Synthesis of the transiently bound Fe/S cluster on the Isu scaffold proteins requires reduced (ferrous) iron and the input of electrons, presumably to generate sulfide (S²⁻) from the cysteine sulfur (S⁰). In a second step, the assembled cluster is transferred from the scaffold to recipient apoproteins. We first describe the Nfs1/Isd11 complex and the Isu scaffold proteins; then we summarize the other components required for transient Fe/S cluster synthesis on the Isu scaffold.

The Cysteine Desulfurase Complex Nfs1/Isd11

The eukaryotic cysteine desulfurase Nfs1 and its bacterial homologs NifS, IscS, and SufS belong to the subgroup of aminotransferases of the pyridoxal 5-phosphate (PLP)-dependent family of enzymes, which catalyze the conversion of cysteine to alanine and sulfane sulfur via the formation of a protein-bound cysteine persulfide intermediate (Figure 2) (Zheng et al. 1993, Mühlenhoff et al. 2004, Kessler 2006). Eukaryotic Nfs1 is highly conserved from yeast to humans and performs an essential function in biogenesis (Land & Rouault 1998, Nakai et al. 1998, Kispal et al. 1999, Li et al. 1999b, Biederbick et al. 2006). The catalytic cycle of cysteine desulfuration by NifS-like proteins is initiated by the formation of a protein that shows similarity to iron-only hydrogenases of bacteria and algae (Table 1). In addition, biogenesis of cytosolic and nuclear Fe/S proteins depends on the mitochondrial ISC assembly system (Figure 1). As we explain in detail below, the organelles appear to export a component to the cytosol that is essential for extramitochondrial Fe/S protein maturation. The central component of this so-called ISC export machinery is a mitochondrial transporter of the ATP binding cassette (ABC) family (Table 1) (Kispal et al. 1999). To date, the eukaryotic ISC assembly, ISC export, and CIA machineries encompass 21 components, impressively underlining the complexity of this allegedly simple biosynthetic process. Nevertheless, under certain conditions, biosynthesis can be accomplished by just two proteins. The NIF proteins NifS and NifU of the eukaryotic parasite Entamoeba histolytica were shown to replace both the ISC and SUF systems of Escherichia coli under anaerobic conditions (Ali et al. 2004).
Schiff base of the amino function of the cysteine substrate with the PLP cofactor (Zheng & Dean 1994, Zheng et al. 1994, Kaiser et al. 2000, Cupp-Vickery et al. 2003). A highly conserved active-site cysteine residue close to the C terminus of the protein then acts as a nucleophile to attack the γ-sulphhydryl group of the cysteine intermediate, resulting in the abstraction of γ-SH from the cysteine intermediate and the formation of a persulfide on the active-site cysteine. The mechanism of sulfur transfer from Nfs1 to the Isu proteins and the subsequent assembly into an Fe/S cluster has not been firmly established. Given its extreme toxicity, sulfur is unlikely to be transferred in free (soluble) form. Rather, sulfur may be handed over from the active-site cysteine of Nfs1 to an accepting catalytic cysteine residue on the Isu proteins (Agar et al. 2000c; Smith et al. 2001, 2005; Urbina et al. 2001).

In support of a direct transfer mechanism, the formation of complexes between Isu1/IscU and Nfs1/IscS has been demonstrated both in vivo and in vitro (Yuvaniyama et al. 2000, Urbina et al. 2001, Gerber et al. 2003). It is unclear, however, whether the formation of a heterodisulfide-bridged intermediate between Isu1 and Nfs1 is involved (Kato et al. 2002) or whether the reaction occurs via transpersulfuration similar to that proposed for sulfur transfer from SuSF to SuFE, which forms a tight complex (Loiseau et al. 2003, Outten et al. 2003). SuFE contains a strictly conserved cysteine residue that binds the sulfur released from cysteine by SuFS as an intermediate persulfide before its further transfer to the scaffold SuFA.

Eukaryotic Nfs1 proteins are unique in that they are firmly associated with Isd11, an essential protein in yeast with no obvious sequence relatives in bacteria (Figure 2) (Adam et al. 2006, Wiedemann et al. 2006). Yeast Nfs1 (50 kDa) and Isd11 (11 kDa) form a stable complex of ~200 kDa. Even though Isd11 is not required for desulphurase activity of Nfs1 (sulfide release) in vitro, the Nfs1/Isd11 complex seems to be the active desulphurase in vivo. Depletion of Isd11 in yeast cells results in a general Fe/S assembly defect and, more specifically, in the loss of Fe/S cluster formation on the Isu1 protein. The situation seems similar to the above-mentioned transpersulfuration reaction within the bacterial SuFS/SuFE complex (Loiseau et al. 2003, Outten et al. 2003). However, a function of Isd11 equivalent to SuFE as an intermediate sulfide carrier can be ruled out because the protein lacks (conserved) cysteine residues. In the absence of Isd11, the Nfs1 protein is prone to aggregation and degradation (Adam et al. 2006, Wiedemann et al. 2006). Therefore, one function of Isd11 is that of a stabilizing component preventing the aggregation of Nfs1.

Besides their role in Fe/S cluster formation, PLP-dependent cysteine desulfurases in bacteria provide sulfur for the biosynthesis of thiamine, thionucleosides of tRNA, and NAD⁺ (Kambampati & Lauhon 2000, Lauhon & Kambampati 2000, Schwartz et al. 2000, Nilsson et al. 2002). In eukaryotes, a comparable role for Nfs1 in thioridine modification of tRNA in both mitochondria and the nucleus has been firmly established (Mühlenhoff et al. 2004, Nakai et al. 2004). The detailed mechanisms of these processes are not understood and seem to differ from those described in bacteria, as additional biosynthetic proteins identified in bacteria are not conserved in eukaryotes (Pierrel et al. 2002, Kambampati & Lauhon 2003, Leipuviene et al. 2004). The thiomodification of tRNA in the nucleus seems to be accomplished by small amounts of Nfs1 that have been identified in this location in yeast and human cells (Land & Rouault 1998, Nakai et al. 2001) (see below). It is an interesting question whether Isd11 is also needed in this process and thus is located also outside mitochondria. As discussed below, nuclear Nfs1 does not seem to perform a function in cytosolic/nuclear Fe/S protein biogenesis, at least not independently of the mitochondrial version of Nfs1.
The Isu Scaffold Proteins

In the ISC systems of bacteria and eukaryotes, the IscU/Isu1 proteins function as molecular scaffolds (Table 1) (Garland et al. 1999, Schilke et al. 1999, Agar et al. 2000a, Urbina et al. 2001, Mühlenhoff et al. 2003a, Tong and Rouault 2006). These low-molecular-mass proteins are related to the N-terminal domain of the NifU protein, the central scaffold protein of the NIF system, and are among the most-conserved proteins in evolution (Johnson et al. 2005). The transient formation of an Fe/S cluster on IscU/Isu1 and the N-terminal domain of NifU has been observed both in vitro and in vivo (Nishio & Nakai 2000, Yuvaniyama et al. 2000, Wu et al. 2002, Mühlenhoff et al. 2003a, Smith et al. 2005). U-type proteins are dimers (Figure 2) and have a compact globular, barrel-shaped structure composed of antiparallel β-strands and α-helices (Ramelot et al. 2004). Three phylogenetically invariant cysteine residues involved in Fe/S cluster formation are located at one end of the barrel close to the surface. The cysteine residues are partially or fully solvent accessible and are located sufficiently close to each other to form a binding site for iron and/or Fe/S clusters. This solvent-exposed Fe/S cluster–binding site on the surface of the protein is compatible with the idea of a scaffold function. Bacterial IscU (and the corresponding NifU segment) is able to assemble two [2Fe-2S] clusters per dimer (Agar et al. 2000a, Yuvaniyama et al. 2000, Smith et al. 2005). After their reductive coupling, a [4Fe-4S] cluster per dimer is formed, suggesting a bridged binding of the [4Fe-4S] cluster between two monomers.

The (bio)chemical mechanism of Fe/S cluster synthesis is not clear, in particular because it is difficult to establish whether sulfur or iron binding occurs as a first step (see Fontecave et al. 2005, Johnson et al. 2005). Presumably, the answer to this question depends on in vitro systems not only involving the Nfs1 and Isu proteins but also additional ISC proteins required for Fe/S cluster synthesis on the Isu scaffold (Figure 3). We discuss these proteins further below.

THE REQUIREMENT OF THE ELECTRON TRANSFER CHAIN FERREDOXIN AND FERREDOXIN REDUCTASE

A crucial role of the [2Fe-2S] ferredoxin Yah1 and its bacterial homolog termed Fdx was documented early on in the elucidation of Fe/S protein biogenesis (Table 1) (Takahashi & Nakamura 1999, Lange et al. 2000). Depletion of these proteins or of ferredoxin reductase Arh1 drastically affects the maturation of Fe/S proteins in vivo (Li et al. 2001b). Subsequent studies in yeast have shown a defect in the de novo assembly of the transient Fe/S cluster on Isu1 in the absence of Yah1 in vivo (Figure 3) (Mühlenhoff et al. 2003a). Apparently, Yah1 (and presumably Arh1) functions early in Fe/S cluster formation. These proteins may serve as an electron transfer chain, providing electrons for the reduction of sulfur (S0) formed in the cysteine desulfurase reaction to sulfide (S2−) present in Fe/S clusters (Figures 2 and 3). However, functions later in biogenesis are not excluded, e.g., in reductive coupling of two [2Fe-2S] to one [4Fe-4S] cluster on the IscU/Isu proteins (see above). In vitro Fe/S protein–reconstitution assays using isolated mitochondria demonstrate that NADH serves as the preferred electron donor for the two proteins (Mühlenhoff et al. 2002a). Further progress toward the mechanistic understanding of the function of this electron transport chain depends on an in vitro system faithfully reproducing its requirement. The putative mammalian counterparts of Yah1 and Arh1 are adrenodoxin and adrenodoxin reductase, respectively. Their well-established function is in steroid biogenesis in mitochondria, suggesting a dual function of these proteins in mammals.
A current working model for the function of the ISC (iron-sulfur cluster) assembly machinery in mitochondria. Biogenesis of Fe/S proteins can be divided in early steps, needed for the synthesis of a transient Fe/S cluster on the scaffold proteins Isu1 and Isu2, and late steps, involving Fe/S cluster transfer and incorporation into target apoproteins. The process starts with the membrane potential (pmf)-dependent import into mitochondria of ferrous iron ($Fe^{2+}$), which is facilitated by the carrier proteins Mrs3 and Mrs4 and further unknown factors (X). Iron binding to the Isu1/Isu2 proteins may be supported by the putative iron donor Yfh1 (frataxin), which directly binds to Isu1. Sulfur is released from cysteine by the Nfs1/Isd11 complex (see Figure 2). This step presumably needs the electron transfer chain NADH $\rightarrow$ ferredoxin reductase Arh1 $\rightarrow$ ferredoxin Yah1 for the reduction of sulfur. ISC proteins required later in the process are the Hsp70 chaperone Ssq1; its cochaperone Jac1, which facilitates the ATP-dependent binding of Ssq1 to Isu1/2; the nucleotide exchange factor Mge1; and the glutaredoxin Grx5. Isa1 and Isa2 form a dimer and are specifically required for functional activation of aconitase-type Fe/S proteins. The role of Nfu1 has not been deciphered. Known protein interactions are indicated. Iron is transferred directly to ferrochelatase (Hem15) to be used in heme synthesis from protoporphyrin IX (PPIX).

THE ROLE OF Mrs3/Mrs4 AND Yfh1 IN TRAFFICKING IRON TO THE Isu SCAFFOLD PROTEINS

The path of iron from the cytosol to the Isu scaffold proteins is still not entirely clear, despite considerable progress over the past few years. In this section, we review some recent studies that have led to a current working model implicating a role of the related mitochondrial carrier proteins Mrs3 and Mrs4 and matrix Yfh1 (yeast frataxin homolog) in iron trafficking (Figure 3). Further studies will have to test this model. The import of iron across the mitochondrial inner membrane occurs in the ferrous form ($Fe^{2+}$) and requires a membrane potential (Lange et al. 1999). Mrs3 and Mrs4 were originally isolated as multicopy suppressors of intron splicing defects and are well conserved in eukaryotes, including mammals (Wiesenberger et al. 1991, Li et al. 2001a). Expression of MRS4 is induced
upon iron deprivation (Shakoury-Elizeh et al. 2004), and the deletion of both genes is associated with an increased cellular iron uptake, the induction of iron-regulated genes, and increased vacuolar iron storage (Foury & Roganti 2002, Nyhus et al. 2002, Mühlenhoff et al. 2003b, Li & Kaplan 2004). Biochemical analysis revealed a clear correlation between the expression levels of these transporters and the efficiency of mitochondrial Fe/S protein and heme biosynthesis in vivo and in isolated mitochondria (Mühlenhoff et al. 2003b, Zhang et al. 2005). Because mitochondrial extracts of Mrs3/Mrs4-deficient cells showed wild-type levels of heme or Fe/S protein formation, it is reasonable to assume an important role for the Mrs3/Mrs4 carriers in iron import into mitochondria. Whether this function is direct (as iron transporters) or indirect (e.g., as regulators) requires the functional reconstitution of purified proteins. Similarly, the form in which iron is transported (free or a chelated ion) is unclear. Double deletion of MRS3 and MRS4 is associated with only mild phenotypes, indicating that other mitochondrial iron-uptake systems must exist. Detailed analysis of the kinetics of heme formation strongly supports this conclusion (Zhang et al. 2005).

Several recent results indicate that Yfh1, the yeast homolog of human frataxin, performs a role in Fe/S protein biogenesis by facilitating iron delivery to the Isu proteins. Frataxin/Yfh1 deficiency in yeast, conditional knockout mice, human cell culture, or flies is associated with impaired Fe/S protein biogenesis (Puccio et al. 2001, Chen et al. 2002, Duby et al. 2002, Mühlenhoff et al. 2002b, Stehling et al. 2004, Anderson et al. 2005). Therefore, a function for Yfh1 in Fe/S protein biogenesis seems well established. In support of these phenotypical investigations, yeast Yfh1 interacts in an iron-stimulated manner with Isu1 (and Nfs1) in vivo, and its depletion is associated with a specific defect in the de novo Fe/S cluster formation on Isu1 (Gerber et al. 2003, Mühlenhoff et al. 2003a).

Similarly, in vitro studies have shown that Yfh1 is able to bind several atoms of iron with low affinity and stimulate Fe/S cluster formation on Isu1 (Yoon & Cowan 2003, Bou-Abdallah et al. 2004, Nair et al. 2004). A proposed high-molecular-mass form seems unnecessary for its functionality (Aloria et al. 2004). Hence, Yfh1 may function as an iron donor in the biosynthesis of Fe/S clusters on Isu1 (Figure 3). Recently, Vivas et al. (2006) reported the requirement of Salmonella CyaY, the bacterial homolog of Yfh1, in the maturation of respiratory complexes I and II, when other genes were compromised. In vitro studies showed an interaction of E. coli CyaY with IscS, and an iron donor function for CyaY during Fe/S cluster synthesis on IscU (Layer et al. 2006). These findings suggest that frataxin may also be involved in bacterial Fe/S protein biogenesis, even though the cyaY gene is not part of the isc operon, and no conspicuous phenotypes are found upon the single deletion of cyaY in E. coli (Li et al. 1999a).

The deletion of yeast YFH1 is associated with comparatively mild effects, indicating that its function can be bypassed (Table 1) (Babcock et al. 1997, Foury & Cazzalini 1997, Wilson & Roof 1997). It is not known whether iron binds spontaneously to Isu1 under Yfh1-deficient conditions or whether other proteins can partially take over its function. In higher eukaryotes, frataxin function is more critical for cell viability, as deletion of its gene in mice is embryonically lethal, and the reduction of its levels by >70% in humans leads to the neurodegenerative disease Friedreich’s ataxia (Campuzano et al. 1996, Cossee et al. 2000). Several other functions of frataxin have been proposed; these include iron export from mitochondria, iron storage, protection from oxidative stress, heme biosynthesis, and the regulation of respiration. Because these suggested functions are beyond the scope of this overview, we refer the reader to other reviews on frataxin (Gerber & Lill 2002, Puccio & Koenig 2002, Wilson 2003).
**Nfu1 AND Isa1/Isa2 PROTEINS: ALTERNATIVE Fe/S SCAFFOLDS OR IRON CHAPERONES?**

The mitochondrial protein Nfu1 exhibits sequence similarity to a 60-residue-long segment of the C-terminal domain of bacterial NifU (Schilke et al. 1999, Johnson et al. 2005). This region contains two conserved cysteine residues and, in NifU, is capable of assembling a [4Fe-4S] cluster that can be transferred to the apoform of nitrogenase in vitro, suggesting that this domain may function as a scaffold (Yuvaniyama et al. 2000, Smith et al. 2005). However, mutations in the C-terminal cysteine residues do not interfere with the maturation of the nitrogenase in vivo (Agar et al. 2000b). The human Nfu1 protein has been shown to bind a labile Fe/S cluster in vitro (Tong et al. 2003). Whether Nfu1 binds Fe/S clusters in vivo remains to be demonstrated. In yeast, NFU1 may be deleted without obvious consequences, but simultaneous deletion with several other ISC genes (e.g., ISU1 and SSQ1) is lethal (Table 1), indicating an auxiliary function of Nfu1 in Fe/S cluster maturation (Figure 3) (Schilke et al. 1999).

In contrast, the deletion of NifU-like proteins in chloroplasts of Arabidopsis thaliana results in slow growth and a significant loss of function of specific chloroplast Fe/S proteins (Leon et al. 2003, Touraine et al. 2004, Yabe et al. 2004, Balk & Lobreaux 2005, Kessler & Papenbrock 2005). In cyanobacteria the deletion of Nfu1 is lethal, whereas genetic ablation of IscA and SufA is not associated with drastic consequences (Balasubramanian et al. 2006). These findings suggest an important function of Nfu1 in these organisms. Recombinant Nfu1-like proteins from plants and cyanobacteria form dimers. The purified proteins can assemble Fe/S clusters that can be transferred to apoferredoxin, but not other Fe/S proteins in vitro, indicating that Nfu1-like proteins of plastids and cyanobacteria may serve as important scaffolds for Fe/S cluster synthesis (Nishio & Nakai 2000). Given the limited sequence homology, the significance of these findings for mitochondrial Nfu1 remains to be elucidated.

All three bacterial systems involved in cellular Fe/S protein formation contain a member of the IscA/Isa1 protein family. *S. cerevisiae* carries two homologous genes termed *ISAI* and *ISAI2* encoding mitochondrial matrix-located proteins (Table 1) (Jensen & Culotta 2000, Kaut et al. 2000, Pelzer et al. 2000). The depletion of yeast Isa1 or Isa2 is associated with defects in the maturation of various Fe/S proteins. Double deletion of both genes does not exacerbate these defects, indicating that the two isoforms perform an overlapping function. In fact, Isa1 and Isa2 form a complex in vivo (U. Mühlenhoff, unpublished data). A human *ISAI* ortholog complementing the yeast mutant has been identified (Cozar-Castellano et al. 2004). All members of the Isa1/Isa1 family contain three conserved cysteine residues that are essential for function in vivo. The strict conservation suggests these proteins may bind iron or Fe/S clusters. Fe/S cluster association to *Schizosaccharomyces pombe* Isa1 was observed in vitro (Wu et al. 2002), yet studies in vivo suggest that *S. cerevisiae* Isa1 and Isa2 bind iron but not an Fe/S cluster (U. Mühlenhoff, unpublished data). A thorough analysis revealed that the proteins are specifically required for the maturation of mitochondrial aconitase–like proteins (*Aco1* and *Lys4* in *S. cerevisiae*), whereas other [2Fe-2S] and [4Fe-4S] cluster–containing proteins were assembled normally. These data satisfactorily explain the deletion phenotypes, e.g., the auxotrophies for glutamate and lysine. Strikingly, the assembly of aconitase–like proteins additionally required the Isu1/Isu2 scaffold. This finding suggests that the Isa1/Isa2 function is specifically needed for a subset of Fe/S proteins, yet the Isa1/Isa2 proteins do not serve as alternative scaffolds in vivo (Figure 3). The specific Isa1/Isa2 function may be the (re)addition of the fourth iron to the cluster of aconitase because this iron ion is not coordinated by protein ligands (Beinert et al. 1997). However, no [3Fe-4S] cluster accumulation was detectable upon the depletion.
of these proteins in vivo (A. Pierik, unpublished data). The specific function of Isa1/Isa2 in aconitase maturation therefore remains to be elucidated.

Whereas Fe/S cluster binding in vivo has not been observed for yeast Isa1/Isa2, the bacterial IscA-like proteins may well serve such a scaffold function. Both Fe/S cluster and iron binding have been reported for bacterial IscA-like proteins in vitro (Krebs et al. 2001; Ollagnier-de-Choudens et al. 2001, 2003; Wollenberg et al. 2003; Ding et al. 2004). Which of the proposed functions may be most relevant for a living cell now requires studies in vivo. Crystal structures of the apo-forms of bacterial IscA and SufA show that the most N-terminal of three conserved cysteine residues is located within a basket-like depression at the protein surface of a protein dimer (Bilder et al. 2004, Cupp-Vickery et al. 2004, Wada et al. 2005). These baskets form a central cavity at the interface of two dimers, which may harbor a solvent-exposed Fe/S cluster (or bound iron). In all three crystal structures, the two C-terminal conserved cysteine residues are disordered, and their exact positions could not be determined. Computer modeling of these residues suggests they may be positioned sufficiently close to each other to accommodate a [2Fe/2S] cluster between two adjacent monomers (Cupp-Vickery et al. 2004). Solving the structure of the holoform of these proteins is a future challenge.

**A DEDICATED MITOCHONDRIAL CHAPERONE SYSTEM FOR CELLULAR Fe/S PROTEIN ASSEMBLY**

In yeast mitochondria, Ssq1 (a specialized chaperone system encompassing the Hsp70), its cognate DnaJ-type cochaperone Jac1, and the nucleotide exchange factor Mge1 play a central role in Fe/S protein biogenesis (Table 1) (Strain et al. 1998, Schilke et al. 1999). *JAC1* and *MGE1* are essential genes in *S. cerevisiae*, whereas the deletion of *SSQ1* is associated with a severe growth phenotype that can be suppressed by the overexpression of *SSC1* encoding the major Hsp70 chaperone of yeast mitochondria (Kim et al. 2001, Voisine et al. 2001, Craig & Marszalek 2002). This indicates that Ssc1 and Ssq1 partially overlap in function, a view supported by several eukaryotic species, including humans, that do not contain a close homolog of Ssq1.

Genes homologous to *SSQ1* and *JAC1* reside in the bacterial *isc* operons, suggesting that the requirement of the encoded chaperones is highly conserved (Table 1) (Zheng et al. 1998). Despite their similar cellular functions, there are several differences between the bacterial and mitochondrial systems (Dutkiewicz et al. 2003). Ssq1 essentially requires the ADP/ATP nucleotide exchange factor Mge1 for function because, in contrast to bacterial HscA, dissociation of Ssq1-bound ADP is rate limiting (Silberg et al. 2000, Dutkiewicz et al. 2003). So far, Isu1 is the only substrate of Ssq1. Ssq1 and Isu1 form a binary complex mediated by a highly conserved recognition motif (LPPVK) within Isu1 that is located within a solvent-exposed loop close to the Fe/S cluster assembly site of Isu1 (Hoff et al. 2000, Dutkiewicz et al. 2003). Synthetic peptides corresponding to this recognition motif stimulate the ATPase activity of HscA with similar affinity as IscU. This peptide specifically binds in an extended conformation to a solvent-accessible hydrophobic cleft in the β-subdomain of HscA (Täpley & Vickery 2004). Alterations in this motif disrupt the interaction with Ssq1/HscA and result in deleterious in vivo phenotypes in *S. cerevisiae* (Dutkiewicz et al. 2004). Conversely, Ssq1-mutant proteins with severely reduced affinities for the Isu proteins...
display similar phenotypes as \textit{SSQ1}-deletion mutants, underscoring the physiological importance of the specific Ssq1-Isu1 interaction (Knieszner et al. 2005). The cycle of Ssq1-Isu1 interaction is similar to that of any Hsp70 with client proteins (for a review, see Craig & Marszalek 2002). The Isu1 protein encounters the ATP-bound form of Hsp70, and binding is stabilized upon hydrolysis of ATP. The cycle of interaction is completed when ADP is exchanged for ATP, and the Isu1 substrate is released. Thus, ATP binding and hydrolysis stimulated by a J-protein are required for function, as expected of any Hsp70 machinery in vivo. Jac1 facilitates complex formation between Ssq1 and Isu1 by specifically binding to Isu1 via its C-terminal domain and escorting it to Ssq1, but it is not essential for the Ssq1-Isu1 interaction (Andrew et al. 2006).

Although recent studies indicate a function of the chaperone system late in biogenesis (Figure 3), the precise mechanistic role of this chaperone system in Fe/S protein biogenesis is not clear. In yeast, the depletion of both Ssq1 and Jac1 causes an accumulation of Fe/S clusters on Isu1 in vivo (Mühlenhoff et al. 2003a). In vitro, Ssq1 and Jac1 did not promote Fe/S cluster assembly on Isu1 (Dutkiewicz et al. 2006). Mutant Isu1 proteins with alterations in the LPPVK recognition motif were capable of Fe/S cluster assembly in vivo but failed to transfer their Fe/S cluster to recipient apoproteins. Together, these findings strongly suggest that these chaperones are required for steps following the de novo synthesis of the Fe/S clusters on the Isu scaffold proteins, for instance, in Fe/S cluster transfer or in recycling of the Isu scaffold. However, this conclusion contrasts with in vitro findings showing that bacterial IscU is capable of efficient Fe/S cluster transfer to apoproteins without further need of chaperones (see above). Resolution of this discrepancy requires an in vitro system in which the molecular function of the chaperones can be faithfully resolved.

\section*{THREE MACHINERIES INVOLVED IN THE MATURATION OF CYTOSOLIC AND NUCLEAR Fe/S PROTEINS}

In contrast to the process described above, the maturation of Fe/S proteins in the cytosol and nucleus is less well understood. Nevertheless, research in the past five years has led to the identification of a number of components involved in the process and to the elucidation of a few general principles underlying biogenesis of Fe/S proteins in these compartments. It is clear from numerous accounts that mitochondria, and in particular the mitochondrial ISC assembly machinery, play a decisive role in the maturation of both mitochondrial and cytosolic/nuclear Fe/S proteins (Figure 4) (see, e.g., Kispal et al. 1999, Lange et al. 2000, Li et al. 2001b, Gerber et al. 2004, Rutherford et al. 2005, Biederbick et al. 2006, Ponderre et al. 2006). In addition, there has been a steady increase in the number of components for which a specific involvement in the biogenesis of cytosolic and nuclear Fe/S proteins has been found. These proteins execute no detectable role in mitochondrial Fe/S protein generation and have been divided in two distinct groups, depending on the phenotypes associated with their functional impairment. (a) Members of the ISC export machinery play a role in both Fe/S protein maturation and the regulation of cellular iron uptake and intracellular distribution (Figure 4). Apparently, the central component of this machinery, a mitochondrial ABC transporter, exports a component to the cytosol, where it is key to both the maturation of cytosolic/nuclear Fe/S proteins and the regulation of iron homeostasis by its interaction with the transcription factors Aft1/Aft2 (see below) (Yamaguchi-Iwai et al. 1996, Blaiseau et al. 2001, Rutherford et al. 2001). These proteins are central factors of metal homeostasis in yeast, determining the expression of the so-called iron regulon (Rutherford & Bird 2004). It is currently unknown whether the transported compounds for both processes are identical or distinct.
Yeast components involved in cytosolic and nuclear Fe/S protein maturation and the role of yeast mitochondria in cellular iron homeostasis. The maturation of cytosolic and nuclear Fe/S proteins crucially involves the mitochondrial ISC (iron-sulfur cluster) assembly machinery (Figure 3). According to the current view, mitochondria synthesize a compound exported to the cytosol by the ATP binding cassette (ABC) transporter Atm1. Other members of the ISC export machinery are the sulfhydryl oxidase Erv1 (which is also involved in protein import) and glutathione (GSH). An Atm1 substrate is also crucial for the regulation of cellular iron uptake by interacting with the Aft1/Aft2 transcription factors. Nonfunctional ISC assembly and export machineries (crossed ISC) cause the translocation of Aft1/Aft2 into the nucleus and the induction of the iron regulon. The maturation of cytosolic/nuclear Fe/S proteins requires the soluble P-loop ATPases (or GTPases) Cfd1 and Nbp35, the iron-only hydrogenase-like protein Nar1, and the seven-bladed WD40 repeat protein Cia1, which interact with each other in vivo in the indicated way. Nbp35 and Nar1 carry Fe/S clusters themselves. Nfs1 performs an essential role in the nucleus, presumably in thiouridine modification of tRNA (tRNA\(^\delta\)). CIA, cytosolic iron-sulfur protein assembly.

Similarly, the chemical nature remains to be elucidated. (b) Members of the CIA machinery, conversely, seem to be specific for the maturation of cytosolic/nuclear Fe/S proteins and have no detectable role in cellular iron homeostasis. To date, four CIA proteins have been identified. We discuss these machineries in more detail below.

**THE MITOCHONDRIAL ISC EXPORT MACHINERY**

The currently known members of the ISC export machinery are the ABC transporter Atm1 of the mitochondrial inner membrane, the intermembrane space protein Erv1, and the tripeptide glutathione (GSH) (Kispal et al. 1999, Lange et al. 2001, Sipos et al. 2002). A hallmark of a yeast strain carrying a deletion in the \textit{ATMI} gene is an auxotrophy for leucine. The search for the underlying reason for this phenotype revealed a specific maturation defect of the cytosolic Fe/S protein Leu1, an isopropylmalate isomerase. Cells in which Atm1 levels are depleted by regulated gene expression maintain growth yet show a substantial defect in the maturation of Fe/S proteins in the cytosol and nucleus but not in mitochondria (Kispal et al. 1999, Balk et al. 2004). Surprisingly, the deletion of \textit{ATMI} is
not lethal as expected from other members of the Fe/S protein assembly systems (Table 1). This may be explained by a functional overlap with Mdl1, another mitochondrial ABC transporter involved in peptide export (Young et al. 2001). Overexpression of MDL1 may restore the defects of ATM1-deletion cells (Chloupkova et al. 2003). Atm1 acts as a dimer (Chloupkova et al. 2004) and, consequently, fewer bacteria contain a close sequence homolog (Lill & Kispal 2001, 2003).

The two human isoforms termed ABCB6 and ABCB7 and the plant protein Sta1 have been shown to replace Atm1 function in yeast (Allikmets et al. 1999, Bekri et al. 2000, Mitsuhashi et al. 2000, Kushnir et al. 2001). This suggests that these proteins perform a similar function as Atm1. A mouse conditional knockout model of ABCB7 directly supports this view (Pondarre et al. 2006). Whereas the deletion of the ABCB7 gene was embryonically lethal in most tissues, deletion in liver resulted in viable animals, yet liver cells showed impaired maturation of the cytosolic Fe/S protein IRP1 and, consequently, a dysregulation of iron homeostasis. Defects in human ABCB7 lead to the rare disease X-linked sideroblastic anemia and cellular ataxia (XLSA/A) (Allikmets et al. 1999, Bekri et al. 2000). That patient cells accumulate iron in mitochondria (the so-called sideroblasts) in this disorder further underscores the functional similarity of the yeast and mammalian proteins (Fleming 2002).

The identification of the Atm1 substrate(s) is one of the major challenges in Fe/S protein biogenesis. Several speculations have been made as to what the substrate might be (Balk & Lill 2004). Reasonable substrates include an Fe/S cluster that is stabilized, e.g., by peptides, a sulfur compound needed for incorporation into cytosolic/nuclear Fe/S clusters, and a compound needed as a cofactor of the CIA proteins discussed below. In an attempt to characterize the Atm1 ABC transporter and its potential substrate, Kuhnke et al. (2006) purified the yeast protein after heterologous expression in bacteria and reconstituted it in proteoliposomes. The ATPase activity was characterized by a $K_m$ of 0.1 mM and a turnover number of $2 \text{s}^{-1}$, which is rather typical for members of the ABC transporter family. Strikingly, the ATPase activity was stimulated specifically by thiol reagents, in particular by peptides containing multiple cysteine residues. Because Atm1 belongs to the group of peptide-translocating ABC transporters, including TAP of the endoplasmic reticulum and Mdl1 (subgroup B of the ABC transporter family) (Dean & Allikmets 2001, Young et al. 2001), one might speculate that the physiological substrate of Atm1 contains free sulfhydryl groups in a peptideic environment.

Similarly, another member of the ISC export machinery, Erv1, also deals with sulfhydryl chemistry (Figure 4) (Lange et al. 2001). This dimeric protein serves as a general sulfhydryl oxidase of the intermembrane space introducing disulfide bridges into target proteins (Lee et al. 2000). The first-known substrate of Erv1 is Mia40, a protein involved in import and folding of the tiny TIM proteins required for the translocation and insertion of carrier and other proteins into the inner membrane (Figure 4) (Mesecke et al. 2005, Rissler et al. 2005). Erv1 may interact with the electron transport chain by shuttling electrons into cytochrome $c$ (Allen et al. 2005, Farrell & Thorpe 2005). The substrate of Atm1, therefore, may be converted into a disulfide-bridged compound in the intermembrane space before exiting to the cytosol. Erv1 belongs to a large family of sulfhydryl oxidases present in virtually all eukaryotes and even in some DNA viruses (Lee et al. 2000, Senkevich et al. 2000, Levitan et al. 2004). In the latter case, the sulfhydryl oxidase encoded by the virus DNA introduces disulfide bridges into viral target proteins in the cytosol to render them competent for virus assembly. The human protein ALR can functionally replace the yeast protein, showing the broad functional conservation of these proteins (Lange et al. 2001). A sequence homolog...
of yeast Erv1, termed Erv2, was identified in the endoplasmic reticulum, where it cooperates with the essential Ero1 enzyme in introducing disulfide bridges into lumenal proteins (Gerber et al. 2001, Sevier et al. 2001). The crystal structure of ALR has been resolved recently and led to the suggestion that the potential mechanism of disulfide formation involves a semiquinone intermediate of the bound flavin adenine dinucleotide cofactor (Wu et al. 2003). Collectively, Erv1 in the intermembrane space may have multiple targets, including a substance that is essential to the formation of Fe/S proteins in the cytosol and nucleus.

The tripeptide GSH is the major redox buffer in yeast and is involved in detoxification processes and protection from oxidative stress (Meister 1995). The depletion of GSH in yeast cells elicits the same specific defect in cytosolic Fe/S proteins and mitochondrial iron accumulation as seen with ATM1- and ERV1-mutant cells (Sipos et al. 2002, Rutherford et al. 2005). This defect is not caused by oxidative damage of the Fe/S clusters in the absence of GSH because mitochondrial Fe/S proteins are functional. Moreover, the phenotypes are also detectable in the presence of dithiothreitol as a reducing agent or under anaerobic conditions. The precise role of GSH in cytosolic/nuclear Fe/S protein maturation remains to be resolved. Nevertheless, GSH may interfere directly with the substrate transported by Atm1. Further progress in the understanding of the role of the ISC export machinery in both Fe/S protein maturation and iron regulation crucially depends on the identification of the substrate(s) of Atm1 and Erv1.

THE CIA MACHINERY

Fe/S protein assembly in the cytosol is assisted by a unique set of proteins that have no primary structural relation to the components of the ISC assembly machinery. To date, functional evidence for four components in cytosolic/nuclear Fe/S protein maturation has been provided in yeast (Figure 4). Because these proteins were discovered during the past three years, we still know little as to how they might function and cooperate in this pathway. For instance, some of the most central aspects of biogenesis are not clear, such as the source of sulfide and iron and how and by which components the Fe/S cluster is assembled. In that respect, it is important to discuss the roles of Nfs1 and Isu1, which both have been found present in small amounts in the cytosol and nucleus of mammalian cells (Land & Rouault 1998, Tong & Rouault 2000). Hence, human Nfs1 and Isu1 may serve as a biogenesis system also outside mitochondria. It is unlikely that the nuclear isoform of yeast Nfs1 is the sulfur donor for cytosolic/nuclear Fe/S proteins owing to studies of yeast mutants in which mitochondrial targeting of Nfs1 was prevented (Kispal et al. 1999, Mühlenhoff et al. 2004, Nakai et al. 2004). No Fe/S protein maturation, neither inside nor outside mitochondria, was seen in this case. Similarly, mitochondrial localization of the Isu proteins in yeast is essential for de novo Fe/S protein maturation in the cytosol, and no Fe/S cluster is assembled on a cytosolic version of Isu1 (Gerber et al. 2004, Mühlenhoff et al. 2004). Therefore, the mitochondrial versions of yeast Nfs1 and Isu1/Isu2 are essential for Fe/S protein maturation in the entire cell.

A similar result was recently obtained for Nfs1 in human HeLa cells (Biederbick et al. 2006). The depletion of endogenous Nfs1 by the RNA interference technique leads to growth arrest and an impairment in the maturation of mitochondrial and cytosolic Fe/S proteins. Heterologous synthesis of murine Nfs1 restores the activities to wild-type levels, yet a cytosolic/nuclear version of murine Nfs1 is incapable of reverting the growth defects and, more importantly, the cytosolic aconitase activity of IRP1. Apparently, Nfs1 in the cytosol/nucleus does not serve as the source of sulfur for Fe/S protein biogenesis, suggesting its major nuclear role in other essential processes such as the thiomodification of tRNAs (see above). Similarly, depletion of the
mitochondrial and cytosolic isoforms of Isu1 in human cells severely affects the aconitase activities in both compartments, whereas a decrease in the cytosolic isoform of Isu1 has no effect on the steady-state activity of cytosolic aconitase IRP1 (Tong & Rouault 2006). Interestingly, a delay in the recovery of cytosolic aconitase activity after treatment with H2O2 or iron chelator was observed in human cells depleted in cytosolic Isu1. This may suggest a function of human cytosolic Isu1 in the regeneration/repair of Fe/S proteins. So far, this process has not been investigated in detail.

Two soluble P-loop ATPases were discovered as central components of the assembly process. Both are essential components in yeast (Table 1). Cfd1 was the first-known cytosolic protein for which an involvement in Fe/S protein biogenesis was shown (Roy et al. 2003). This protein was identified using clever genetic experiments in which researchers studied the efficiency of the maturation of human cytosolic IRP1 in yeast. Later, Hausmann et al. (2005) described a similar protein termed Nbp35 that exhibits high sequence similarity in the middle- and C-terminal parts of Cfd1. Both proteins form an oligomeric complex in vivo (Figure 4) (D.J.A. Netz, unpublished data). At its N terminus, Nbp35 possesses a ferredoxin motif with four conserved cysteine residues that bind an Fe/S cluster (Hausmann et al. 2005). Its maturation requires the mitochondrial ISC assembly and export machineries. The removal of this segment or mutation of the cysteine residues to alanine results in the loss of cell viability and no Fe/S cluster binding, demonstrating the functional importance of this cofactor. Both proteins contain an additional four conserved cysteine residues in their C-terminal half. Mutational studies showed that at least two of these residues are essential for function (Roy et al. 2003). Recent in vitro reconstitution studies have shown that both proteins can assemble an Fe/S cluster on these residues (D.J.A. Netz, unpublished data). These clusters are rather labile and dissociate readily from Cfd1 and Nbp35. The transient character of Fe/S cluster binding suggests that these proteins serve as scaffolds for the transient assembly and binding of Fe/S clusters before their transfer to apoproteins. Support for this hypothesis requires evidence for the binding of Fe/S clusters to Cfd1 and Nbp35 in vivo.

Another central protein of CIA machinery is Nar1, which contains two interacting Fe/S clusters and shows sequence similarity to bacterial iron-only hydrogenases (Table 1) (Balk et al. 2004). These clusters are coordinated by four conserved cysteine residues at the N terminus and another four scattered in the middle- and C-terminal parts of Nar1. It seems unlikely that Nar1 contains a similar C-terminal cluster as hydrogenases (the so-called H-cluster) (Nicolet et al. 2002, Armstrong 2004) because specialized bacterial proteins needed for biogenesis of this H-cluster (containing a [4Fe-4S] cluster and a 2-Fe center) are not conserved in eukaryotes (Posewitz et al. 2004). Electron paramagnetic resonance spectroscopy further supports this notion by showing characteristically different spectra for purified yeast Nar1 and bacterial hydrogenases (Balk et al. 2004, 2005b). Fe/S cluster assembly on Nar1 depends on the mitochondrial ISC assembly and export machineries and on Cfd1 and Nbp35 (Figure 4). This dependence clearly shows that Nar1 is not only an active component of Fe/S protein maturation but also a target Fe/S protein of this pathway, creating a chicken-and-egg situation that is essential for life. Human cells contain two homologous proteins (Barton & Worman 1999). Because neither of them complements yeast Nar1-depletion mutants, it remains unclear whether these proteins are functional orthologs; hence, direct functional studies in human cells are necessary.

The fourth-known protein of the CIA machinery, termed Cia1, has somewhat different characteristics than Cfd1, Nbp35, and Nar1 (Balk et al. 2005a). Whereas the depletion of Cia1 in yeast results in a strong impairment of Fe/S cluster incorporation into target proteins such as cytosolic Leu1 and Rli1, or...
nuclear Ntg2, the Fe/S clusters of the CIA components Nbp35 and Nar1 are assembled normally in the absence of Cia1. Evidently, during Fe/S protein maturation on target proteins, Cia1 acts after the assembly of the Fe/S clusters on Nbp35 and Nar1. Even though a more detailed molecular view of Cia1 function requires in vitro experiments, the protein may play some role in the final incorporation of the Fe/S clusters into true target proteins. Cia1 belongs to the large family of WD40 proteins and exhibits a seven-bladed propeller structure (V. Srinavasan, D.J.A. Netz, A.J. Pierik, R. Lill, H. Michel, unpublished data). These proteins function in rather diverse processes and generally act as protein-interaction devices (Smith et al. 1999). In this context, it is important to mention that Cia1 interacts with Nar1 in vivo (Figure 4) (Balk et al. 2005a). Even though the CIA1 and CFD1 genes are fused in Schizosaccharomyces pombe, suggesting a functional cooperation of the two encoded proteins, a protein interaction between Cia1 and Cfd1 has not been found in S. cerevisiae.

Whereas Cfd1, Nbp35, and Nar1 are predominantly located in the cytosol with minor amounts residing in the nucleus, the situation for Cia1 is reversed (Balk et al. 2005a). Moreover, Cia1 seems much more abundant than the other three proteins, which are present in only small amounts (Ghaemmaghami et al. 2003). Thus, Cia1 may have additional functions in the nucleus. As the human homolog termed Ciao1 possibly performs a role in transcription regulation, a similar function of yeast Cia1 is conceivable (Johnstone et al. 1998). Fe/S protein assembly in the cytosol/nucleus has not been reconstituted in vitro, neither with cytosolic extracts nor with isolated proteins. Such an achievement is a prerequisite for further progress in our understanding of the biochemical course of events in biogenesis. In all likelihood, further CIA components are required to assemble Fe/S proteins in the cytosol and nucleus. Such components may include factors specifically required for the generation of nuclear Fe/S proteins. To date, the compartment in which cofactor insertion into nuclear Fe/S apoproteins occurs is unknown. This reaction may either occur in the cytosol or take place in the nucleus, involving apoprotein import and assembly by the nuclear fraction of the CIA proteins.

**REGULATORY CROSS TALK BETWEEN Fe/S PROTEIN ASSEMBLY, CELLULAR IRON HOMEOSTASIS, AND THE BIOGENESIS OF HEME**

*S. cerevisiae* mutants with defects in the mitochondrial ISC assembly and export systems display a misregulated cellular iron homeostasis that is manifested by the transcriptional induction of the iron regulon involved in cellular iron uptake and the accumulation of iron within mitochondria (Kispal et al. 1999, Li et al. 1999b, Foury & Talibi 2001, Chen et al. 2004, Lange et al. 2004). The latter process seems to be a pathological status, as under normal conditions the yeast vacuole serves as the physiological iron reservoir (Li & Kaplan 2004). Similarly, two iron storage diseases are associated with mutations in ISC components, namely Friedreich’s ataxia and XLSA/A (see above). The iron regulon contains a set of ~30 genes with products that function in iron acquisition (such as FET3, FTR1, FRE1, and FRE2), iron-siderophore uptake (ARN1-ARN4 and FIT1-FIT3), and intracellular iron distribution to vacuoles and mitochondria (FET5, FTH1, and MRS4). These genes are regulated by the iron-sensing transcriptional activators Aft1 (and possibly Aft2) (for a review, see Rutherford & Bird 2004). Aft1 shuttles between the nucleus in iron-deficient cells and the cytoplasm in iron-replete cells (Yamaguchi-Iwai et al. 2002, Ueta et al. 2003), depending on the activity of the mitochondrial ISC assembly and export machineries (Figure 4). Thus, in yeast, iron availability seems to be sensed via the activity of the mitochondrial Fe/S protein assembly pathways independently of the cytosolic iron levels (Chen et al. 2004, Rutherford et al. 2005). However,
regulation of Aft1/Aft2 is not mediated via a canonical cytosolic Fe/S protein, as yeast cells defective in members of the CIA machinery fail to induce the iron regulon (Balk et al. 2004, 2005a; Hausmann et al. 2005). These findings suggest that the mitochondrial ISC assembly and export machineries contribute to the production and export of a key signaling molecule for the expression of iron-regulated genes in the nucleus (Figure 4). Although the regulation of transcription by Aft1/Aft2 represents a central mechanism of cellular iron acquisition, the signal produced and transported by the ISC systems remains elusive.

The major iron-metabolizing processes in the cell are the biosyntheses of heme and Fe/S clusters by pathways thought to occur independently. However, accumulating evidence suggests that there is an intimate mutual cross talk between these two pathways. For instance, the synthesis of heme A of cytochrome oxidase requires the ISC assembly protein Yah1 (Barros et al. 2002). Furthermore, the disruption of mitochondrial ISC assembly and export systems in yeast is generally associated with a decreased heme biosynthesis and cytochrome deficiency, even though this seems to be a secondary (late) consequence (Kispal et al. 1997, Lesuisse et al. 2003, Lange et al. 2004, Zhang et al. 2005). Ferrochelatase (yeast Hem15 in Figure 3), the enzyme catalyzing the last step of heme biosynthesis, is reversibly inhibited in yeast cells with defects in the biosynthesis of mitochondrial Fe/S proteins. This deficiency was first observed in yeast yfh1 mutants, suggesting a direct functional link between Yfh1 and ferrochelatase, but later was found to be a general phenotype of all cells with defective ISC assembly components. Consistent with these observations, no direct interaction between Yfh1 and ferrochelatase is detectable in vivo (Lange et al. 2004).

The molecular mechanisms of the regulatory connection between mitochondrial ISC assembly and heme biosynthesis in S. cerevisiae are not fully understood yet, but two different forms of interplay have been determined experimentally. First, mitochondria with disrupted mitochondrial ISC assembly accumulate an unknown regulatory molecule that causes reversible inhibition of ferrochelatase (Lange et al. 2004, Zhang et al. 2005). Second, transcription of HEM15 encoding ferrochelatase is repressed under low-iron conditions (Shakoury-Elizeh et al. 2004, Puig et al. 2005). A similar repression is observed in cells with defects in mitochondrial Fe/S protein biogenesis (A. Hausmann, B. Samans, R. Lill, U. Mühlenhoff, unpublished data). Presumably, the link between these two major iron-utilizing processes in part may be mediated through the dysregulation of iron homeostasis caused by defective mitochondrial Fe/S protein assembly. Wingert et al. (2005) detected a similar regulatory connection in a zebrafish grx5 mutant, yet with a fundamentally different underlying mechanism. This defect in Grx5 elicits hypochromic anaemia, i.e., a strong defect in heme biosynthesis, and was shown to be mediated via the cytosolic Fe/S protein IRP1 (Hentze et al. 2004), which blocks the translation of the messenger RNA for aminolaevulinate synthase 2, a key enzyme of heme biosynthesis. Together, these recent findings establish the intimate connection between heme and Fe/S protein biosynthesis in both lower and higher eukaryotes and indicate that hemoglobin production in red blood cells is likely interconnected to the biogenesis of Fe/S clusters.

CONCLUSIONS AND OUTLOOK

During the past few years, researchers of Fe/S protein biogenesis have been mainly hunters and gatherers for the identification of new components involved in this novel process. Even though this period is being replaced little by little through mechanistic approaches, more components will likely be identified for this complex biosynthetic reaction. The CIA machinery seems especially far from being completely known. Central future directions for Fe/S protein biogenesis include the elucidation of the chemical mechanism of Fe/S
cluster synthesis on the Isu scaffolds, the roles of the ferredoxin electron transport chain and the chaperone system in mitochondrial biogenesis, the exact function of Atm1 in the maturation of extramitochondrial Fe/S proteins, the biochemical characterization of the Atm1 substrate(s), and the mechanistic understanding of the CIA components in the cytosol and nucleus. From the high conservation of the ISC and CIA genes in eukaryotes, it appears that yeast is an excellent model system with which to study this basic biochemical process. Nevertheless, studies in higher eukaryotes are obligatory to define the similarities and differences to the yeast paradigm. In particular, there are substantial differences expected with respect to the connections of heme synthesis and iron regulation, which follow different principles in higher eukaryotes. Elucidation of the mechanistic fundamentals of Fe/S protein biogenesis is also seminal for understanding the pathology associated with diseases caused by the malfunction of this process. Progress in reaching these goals will benefit from the development of new tools to analyze Fe/S protein assembly in living cells.

**SUMMARY POINTS**

1. Biogenesis of Fe/S proteins is an essential and ancient biosynthetic process of all cells.
2. Mitochondria are involved in biogenesis of virtually all cellular Fe/S proteins.
3. Eukaryotic Fe/S protein biogenesis is mediated by three proteinaceous machineries: the mitochondrial ISC assembly system inherited from bacteria, the ISC export apparatus, and the CIA machinery. The latter two systems are specifically involved in the maturation of Fe/S proteins in the cytosol and nucleus.
4. The basic concept of Fe/S protein biogenesis is the transient assembly of an Fe/S cluster on a scaffold protein with sulfur derived from a cysteine desulfurase.
5. Synthesis of the Fe/S cluster requires reduced iron and electrons from a ferredoxin electron transfer chain.
6. A dedicated mitochondrial Hsp70 chaperone system is required after Fe/S cluster synthesis on the Isu1 scaffold.
7. Fe/S protein biogenesis in mitochondria, but not in the cytosol, is intimately linked to heme biosynthesis and the regulation of cellular iron homeostasis.
8. The malfunction of Fe/S protein biogenesis is connected to neurodegenerative diseases.

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Describe the function of the essential Fe/S protein Nar1 in cytosolic and nuclear Fe/S protein biogenesis.


Provides evidence for the important role of mitochondrial Fe/S protein biogenesis rather than cytosolic iron in the regulation of iron uptake into yeast cells.
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Defines the specificity of the previously identified mitochondrial chaperone system comprised of the Hsp70 Ssq1 and the cochaperone Jac1 for Fe/S protein biogenesis.

Defines the function of Nfs1 and Atm1 in the de novo biogenesis of cellular Fe/S proteins in yeast.


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**Demonstrates in vivo staging of the reactions leading to Fe/S protein assembly in the mitochondrial matrix.**

**This article, along with Nikai et al. 2004, provides evidence for the essential role of nuclear Nfs1, presumably in thio-uridine modification of tRNA.**


Shows the important function of the mouse mitochondrial ABC transporter ABCB7 (and thus murine mitochondria) in cytosolic Fe/S protein biogenesis.
Describes the P-loop NTPase Cfd1, the first component of the CIA machinery responsible for Fe/S protein maturation in the cytosol and nucleus.

Provides evidence for the important role of mitochondrial Fe/S protein biogenesis rather than cytosolic iron in the regulation of iron uptake into yeast cells.

Identifies the Isu proteins as important components of the ISC assembly machinery.


**Defines the specificity of the previously identified mitochondrial chaperone system comprised of the Hsp70 Ssq1 and its cochaperone Jac1 for Fe/S protein biogenesis.**

**Demonstrates the importance of Fe/S protein assembly for heme synthesis via regulation of translation of mitochondrial E-ALAS2 by cytosolic IRP1.**


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