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Chloroplast HCF101 is a scaffold protein for [4Fe-4S] cluster assembly

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Abbreviations: HCF, high chlorophyll fluorescence; PSI, Photosystem I; FSC, Fe/S containing P-Loop NTPases; Fe/S, iron-sulfur; NIF, nitrogen fixation; ISC, iron-sulfur-cluster; CIA, cytosolic Fe/S assembly machinery; WT; wild type.

ABSTRACT

Oxygen evolving chloroplasts possess their own iron-sulfur cluster assembly proteins including members of the SUF and the NFU family. Recently, the chloroplast protein HCF101 (high chlorophyll fluorescence) has been shown to be essential for the accumulation of the membrane complex photosystem I and the soluble ferredoxin-thioredoxin reductases, both containing [4Fe-4S] clusters. The protein belongs to the FSC ([4Fe-4S]-cluster) superfamily of P-loop NTPases, several members of which play a crucial role in Fe/S cluster biosynthesis. Although the C-terminal Fe/S cluster binding site conserved in other members of the FSC family is not present in chloroplast HCF101 homologues, using Mössbauer and EPR spectroscopy, we provide evidence that HCF101 binds a [4Fe-4S] cluster. ⁵⁵Fe incorporation studies of mitochondria-targeted HCF101 in *S. cerevisiae* confirmed the assembly of an Fe/S cluster in HCF101 in a Nfs1-dependent manner. Site-directed mutagenesis identified three HCF101-specific cysteine residues required for assembly and/or stability of the cluster. We further demonstrate that the reconstituted cluster is transiently bound and can be transferred from HCF101 to a [4Fe-4S] apoprotein. Together, our findings suggest that HCF101 may serve as a chloroplast scaffold protein that specifically assembles [4Fe-4S] clusters and transfers them to chloroplast membrane and soluble target proteins.

INTRODUCTION

Fe/S (iron-sulfur) clusters are ancient co-factors of proteins required for various essential processes. Being able to transfer electrons and to act as a catalyst they are important players in photosynthesis and respiration, nitrogen and sulfur metabolism, redox regulation and sensing as well as gene expression, as they are indispensable prosthetic groups of many proteins [1-3]. The biogenesis of such clusters from elemental iron and sulfur is an enzymatic process and requires a set of specialized proteins. As a first step sulfur is mobilized from the sulfhydryl group of cysteine catalyzed by a cysteine desulfurase, while elemental iron is relocated from cellular stores [4]. Synthesis of the Fe/S cluster takes place on specialized scaffold proteins, which assemble transiently bound clusters before their transfer to apo-proteins. The components responsible for Fe/S protein maturation have been mostly characterized in eubacteria and yeast where different systems for Fe/S cluster biogenesis have been identified. The ISC (iron-sulfur-cluster) assembly machinery is present in many bacteria and mitochondria [5,6]. The SUF (sulfur-mobilization) system is active under iron-limiting and oxidative stress situations in bacteria and is also found in plastids [7]. The NIF (nitrogen-fixation) machinery is dedicated to the assembly of nitrogenase in nitrogen-fixing bacteria [8]. In eukaryotes the mitochondrial ISC machinery not only supports the organellar demand for the cofactor but is also required for the assembly of cytosolic and nuclear Fe/S proteins. Additionally, proteins of the CIA (cytosolic Fe/S protein assembly) machinery are essential for Fe/S cluster formation [9,10].

Several Fe/S cluster-containing proteins in chloroplasts need a specialized set of proteins for Fe/S cluster synthesis as the oxygen-sensitive biosynthesis process takes place within an organelle with a high oxygen concentration. In recent years some of the participating proteins have been identified [11-14]. A candidate for sulfur mobilization in chloroplasts, CpNifS, has been shown to act as a cysteine desulfurase *in vitro* and *in vivo* [15,16]. This process seems to be stimulated by CpSufE [17]. For the second step in Fe/S cluster assembly the plastid scaffold protein NFU2 was demonstrated to carry a [2Fe-2S] cluster, which can be transferred to ferredoxin. As levels of PSI (Photosystem I) are decreased in mutant plants, NFU2 is proposed to be also involved in the assembly of [4Fe-4S] proteins, although the mechanism of maturation remains unclear [18,19]. Two further plastid scaffold proteins carrying a transient [2Fe-2S] cluster have been identified recently, CplscA and two glutaredoxines, GrxS14 and Grx16, all of which were demonstrated to assemble and transfer [2Fe-2S] clusters *in vitro* [20,21]. An IscA homologue in *Synechocystis*, has been shown to assemble a [2Fe-2S] cluster and to reactivate apo-adenylyl sulfate reductase, which requires a [4Fe-4S] cluster for activity [22]. However, up to date plastid candidates, which specifically assemble and transfer [4Fe-4S] clusters have not been described.

Apart from PSI, which contains three [4Fe-4S] clusters, a number of other chloroplast enzymes depend upon this cofactor, such as the heterodimeric FTR (ferredoxin-thioredoxin reductase) complex, the nitrite and sulfite reductases, and presumably the NAD(P)H dehydrogenase. Two plastid proteins, HCF101 and APO1, have been genetically described as essential and specific factors for assembly of [4Fe-4S] cluster containing protein complexes although the molecular mechanism remained unclear [11,23]. Whereas APO1 function interferes with translation of photosystem I transcripts *psaA* and *psaB*, HCF101 exerts its function on a posttranslational step [23]. In addition to the lack of PSI, the HCF101 mutants accumulate less heterodimeric FTR complex, whereas levels of [2Fe-2S] cluster containing proteins are not affected. APO1 is only conserved among higher plants, [11] whereas HCF101 belongs to the ancient and ubiquitously distributed protein family of soluble P-loop NTPases [23,24]. Four classes of P-Loop NTPases involved in Fe/S cluster biogenesis have been defined, members of which share a highly conserved protein signature and represent bacterial, plastid, mitochondrial, and cytoplasmic proteins [23].

Plastid homologues and eubacterial forms with an N-terminal DUF59 (domain of unknown function, pfam PF01883) domain belong to class I. The mature HCF101 protein without the predicted transit peptide contains an N-terminal DUF59 domain and eight cysteine residues along the sequence. All cysteine residues are conserved among higher plants (Supplementary Fig. 1). Of the two cysteines located in the DUF59 domain only C128 is highly conserved (Supplementary Fig. 1). The eubacterial representatives of class I could further be divided into subclasses, depending on the presence of the DUF59 domain and the positions of the cysteine residues. Of the eubacterial FSC proteins, ApbC in *Salmonella enterica* is required for the maturation of Fe/S clusters proteins present in the thiamine biosynthetic pathway [25,26,27]. Class II members are mitochondria-targeted eukaryotic proteins. Recently, *Yarrowia lipolytica* Ind1 has been shown to function in the assembly of mitochondrial complex I, possibly acting as a scaffold protein for Fe/S cluster assembly [28]. This protein and the *Arabidopsis* homologue AtInd1 (also termed HCF101-L1) are localized in mitochondria and share conserved C-terminal cysteine residues [28,29]. Nbp35 and Cfd1, members of classes III and IV in yeast, have been shown to form a stable complex and to act in [4Fe-4S] cluster assembly in the cytosol [30-32]. They both bind a transient [4Fe-4S] cluster at the C-terminus and Nbp35 binds an additional [4Fe-4S] cluster at the N-terminus [32]. Homologous members of the FSC family in *Arabidopsis*, yeast, and prokaryotes, which belong to classes II-IV share several conserved cysteine residues (Fig. 1) but strikingly, not even one of those is present in plastid HCF101 forms [26,28,30-32]. Furthermore, characteristic motifs for Fe/S cluster binding, such as CXXC are present in the N- and/or C-termini of cytosolic and mitochondrial proteins in eukaryotes and in eubacteria, like the *Salmonella* homologue ApbC, but remarkably are lacking in the plastid HCF101 form (Fig. 1).

In this study we present a detailed biochemical and spectroscopic characterization of HCF101 *in vitro* and *in vivo*. As shown by UV-Vis, EPR and whole-cell Mössbauer spectroscopy HCF101 carries a [4Fe-4S] cluster that can be rapidly transferred to convert the apoprotein form of a yeast [4Fe-4S] protein into a catalytically active holoenzyme. ⁵⁵Fe radiolabelling experiments in yeast further confirmed the incorporation of an Fe/S cluster into HCF101 in a physiological environment. Taken together, we propose a scaffold function for HCF101 that *in vivo* supports the assembly of the [4Fe-4S] clusters into chloroplast PSI and FTR complexes.

EXPERIMENTAL

Protein overexpression and purification

The complete cDNA of HCF101 was obtained from a cDNA library [33]. To express HCF101 without the predicted transit peptide the cDNA fragment corresponding to amino acid residues 64 to 533, including the stop codon, was cloned into the *Nde*I and *Bam*HI restriction sites of pET23a⁺ (Novagen). Plasmids were transformed into *E. coli* strain BL21 (DE3)/pLysS (Novagen), and cells were initially grown at 37°C in LB medium. Overexpression was induced at OD₆₀₀ of 0.6 by the addition of 1 mM IPTG (isopropylthio-β-galactoside), and cells were harvested after 5 h growth at 30°C. Cells were broken in a French press (Microfluidics, Lampertheim, Germany) in a buffer containing 25 mM Tris-HCl, pH 8.1, 4 mM MgCl₂, 10% glycerol and 1 mM DTT, if not otherwise indicated. After removal of the membranes by centrifugation at 22,000 x g for 30 min, DNA was precipitated with 1% streptomycin sulfate and centrifuged as above. The supernatant was applied to a HiPrep 16/10 DEAE Sepharose Fast Flow IEX column (GE Healthcare, Braunschweig, Germany) and eluted with a 0 - 0.5 M NaCl gradient in the same buffer as above. Fractions containing the purified protein were concentrated with Amicon ultracentrifuge units (Millipore, Billerica, USA) and protein concentration was determined with the Bradford reagent (Roth, Karlsruhe,

Germany). Proteins were visualized by 15% Tris-Glycine-SDS-PAGE and Coomassie staining as described.

For Mössbauer analysis of whole cells BL21(DE3)/pLysS cells containing the respective plasmids were grown on M9 minimal medium. ^{57}Fe (Chemotrade, Leipzig, Germany) was prepared as described [34] and added in a final concentration of $50\ \mu\text{M}$ $^{57}\text{FeCl}_2$ 1 h before induction of overexpression. Cells were harvested 7 h after induction and frozen rapidly in liquid nitrogen.

Site directed mutagenesis of all cysteine residues of HCF101

All cysteine codons were exchanged by serine codons in the expression vector using site-directed mutagenesis as described [35]. The oligonucleotides used to insert the point mutations are listed in Supplementary Table 1. The presence of the mutated bases was confirmed by DNA sequencing.

Spectroscopic methods

UV-visible absorption spectra were recorded with a Jasco V-550 spectrometer in a 1 ml quartz cuvette sealed with a rubber stopper. The cuvette contained 1 mg/ml of HCF101 protein in Tris-HCl pH 8.0, 150 mM NaCl (buffer A).

Mössbauer data were recorded on a spectrometer with alternating constant acceleration. The minimum experimental line width was $0.24\ \text{mm s}^{-1}$ (full width at half-height). The sample temperature was maintained constant in an Oxford Instruments Variox cryostat and the $^{57}\text{Co/Rh}$ source (1.8 GBq) was at room temperature. Isomer shifts are quoted relative to iron metal at 300K. The zero-field spectra have been simulated by using Lorentzian doublets.

For EPR analysis purified HCF101 was transferred to EPR tubes (4.7 outer diameter, 0.45 mm wall thickness, Ilmasil-PN high purity quartz, Quarzschmelze Ilmenau, Langewiesen, Germany) directly after reconstitution and desalting. For reduction of the Fe/S cluster of HCF101 a freshly prepared solution of sodium dithionite in buffer A was added to a final concentration of 2 mM. After mixing the sample was capped with rubber tubing stoppered with a perspex rod and shock-frozen in liquid nitrogen. EPR spectra were recorded with a Bruker E500 ELEXSYS continuous-wave X-band spectrometer using a standard ER4102ST cavity and an ESR910 Oxford Instruments helium-flow cryostat. The magnetic field was calibrated with an ER035M Bruker NMR probe and the microwave frequency was measured with a HP5352B Hewlett-Packard frequency counter.

Chemical reconstitution of Fe/S clusters and cluster transfer to apo-Leu1

Purified recombinant apo-HCF101 used for chemical reconstitution contained only approximately 0.2 moles Fe and S per monomer. Before chemical reconstitution of the Fe/S clusters in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) apo-HCF101 ($60\ \mu\text{M}$) was reduced with 10 mM DTT in 25 mM buffer A for one hour. A five molar excess over the HCF101 protein concentration of ferric ammonium citrate and lithium sulfide per HCF101 monomer was added, and the sample was incubated for 30 min at 23°C . Non-bound iron and sulfide was removed by desalting on a 10 ml Sephadex G-25 column (GE Healthcare, Braunschweig, Germany) equilibrated in buffer A. Protein concentration was determined with the Microbiuret method at 545 nm [36]. Iron was determined colorimetrically after treatment with 1% (w/v) HCl at 80°C for 10 min and addition of ferene as iron chelator [37]. Acid-labile sulfur was determined using the methylene-blue method [37]. For Fe/S cluster transfer holo-HCF101 was mixed with recombinant, reduced apo-Leu1 [32]. Aliquots were analyzed at various time points for Leu1 activity.

⁵⁵Fe incorporation

The yeast strains used were *S. cerevisiae* W303 and the galactose-regulable mutant Gal-NFS1 [6]. A construct was generated which fuses the mature HCF101 protein to the F₁- β mitochondrial targeting sequence and a C-terminal Myc tag by cloning into a pRS426 vector. The construct is expressed under the control of the *TDH3* promoter. Additionally, the targeting sequence was excised allowing cytoplasmic expression of HCF101. The oligonucleotides used are listed in Supplemental Table 1. The plasmids were transformed into W303 or into Gal-NFS1 cells. Wild-type (WT) cells were cultivated for 40 h in SC minimal medium containing 2% glucose (m/v). Gal-NFS1 cells were grown in 2% (m/v) galactose or glucose. The cells were cultured in iron-free minimal medium during the last 16 h. After washing and transfer to fresh iron-free minimal medium, ~0.5 g of cells were radiolabeled for 2 h with ⁵⁵FeCl₃. Following radiolabeling, cells were resuspended in 0.5 ml lysis buffer containing 10 mM Tris, pH 7.4, 2.5 mM EDTA, 150 mM NaCl, 10% glycerol (m/v) and 0.5% Triton X-100 (m/v) and disrupted with glass beads. Anti-Myc beads (20 μ l) were added to the lysates and rotated for 1 h at 4°C. After immunoprecipitation, beads were washed thrice with lysis buffer and the amount of HCF101-Myc-associated radioactivity was measured by scintillation counting. Immunoblot analysis of expressed fusion proteins was performed using anti-Myc and anti-Nfs1 antisera [6].

Results

Expression and purification of HCF101 from *E. coli*

To biochemically investigate the function of HCF101 the coding sequence of the mature-sized protein without the predicted transit peptide was cloned into a pET23a⁺ vector and overexpressed without a tag in the *E. coli* strain BL21(DE3)/pLysS. Five hours after induction with IPTG the total amount of HCF101 protein reached approx. 20% of the total soluble *E. coli* protein extract. The protein migrated at an apparent molecular mass of about 53 kDa (calculated mass 50.5 kDa). After purification with anion exchange chromatography the protein was found to be about 90% pure (Fig. 2a). HCF101 was purified under standard aerobic conditions, and the eluate presented a brownish color. UV-visible absorption spectra of the purified protein showed a prominent peak at 330 nm and a discrete shoulder at 420 nm indicating the presence of intact as well partially degraded [4Fe-4S] clusters (Fig. 2b).

Chemical reconstitution of HCF101

For better investigation of the cluster type, the cofactor was chemically reconstituted by anaerobic incubation of the purified HCF101 protein with a fivefold molar excess of iron and sulfide over the HCF101 protein concentration. UV-visible absorption spectra of the sample and determination of the iron and sulfide content showed that the intensity of the characteristic shoulder at 420 nm was increased up to four fold as compared to the non-reconstituted (as isolated) protein (Fig. 2b and c). Dithionite reduction resulted in about 50% bleaching of the absorbance at 400 nm, a behaviour normally associated with [4Fe-4S] clusters. The reconstituted sample contained about 4 iron and 4 sulfur ions bound per monomer, indicating that HCF101 may bind a single [4Fe-4S] cluster per monomer (Fig. 2c). The reconstituted Fe/S cluster proved to be oxygen sensitive, as the 420 nm shoulder slowly decreased after treatment with air and the reconstituted cluster was almost completely lost after 4 h (Supplementary Fig. 2).

Cysteine residues required for binding the [4Fe-4S] cluster in recombinant HCF101

As the location of the cysteine residues in HCF101 differs from all other members of the FSC family and typical Fe/S cluster binding proteins, it was particularly challenging to investigate the cysteine residues involved in binding the Fe/S cluster. As a first approach the ability to bind a cluster was analyzed *in vitro*. For this purpose we mutated all eight cysteine residues to serine by site-directed mutagenesis. The recombinant proteins were expressed and purified as described for the WT protein. The typical brownish color of WT HCF101 was not found for three of the mutant forms (C128S, C347S and C419S). UV-visible spectral analysis confirmed that the characteristic shoulder at 420 nm was completely absent in these mutant proteins (Fig. 2d). All other recombinant proteins showed only a slightly diminished intensity of the shoulder at 420 nm indicating that less Fe/S cluster was associated with these mutant proteins after biosynthesis in *E. coli*, but the Fe/S cluster was still able to bind (data not shown). The involvement of at least three cysteine residues in Fe/S cluster association to HCF101 is compatible with the stoichiometry of one cluster per monomer.

Whole-cell Mössbauer and EPR spectroscopy studies on recombinant HCF101

To further elucidate the nature of the cluster and the cysteine residues involved in binding we performed whole-cell Mössbauer and EPR spectroscopy. The zero-field Mössbauer spectrum recorded with intact *E. coli* cells after overexpression of WT HCF101 in ^{57}Fe enriched media at 80K showed a superposition of two quadrupole doublets with intensity ratio 89:11 (Fig. 3a, upper panel). The moderately low isomer shift, $\delta = 0.50 \text{ mm s}^{-1}$ and quadrupole splitting, $\Delta E_Q = 1.04 \text{ mm s}^{-1}$ of the major species are typical of [4Fe-4S] clusters in the diamagnetic (2+) state. The iron sites, which formally are two Fe^{2+} and two Fe^{3+} ions, are indistinguishable because of complete valence delocalization. The 11% minority subspectrum, in contrast, has a very large isomer shift, $\delta = 1.26 \text{ mm s}^{-1}$ and large quadrupole splitting, $\Delta E_Q = 3.23 \text{ mm s}^{-1}$, which are typical of high-spin Fe(II) with six hard ligands. Such a component has been observed in whole cells previously and could be assigned to primary intermediates of iron metabolism [34,35].

The Mössbauer spectrum of the C128S mutant control sample shows essentially also two quadrupole doublets (Fig. 3a, lower panel), which have rather broad lines and resemble neither the spectra known for heme enzymes nor those of Fe/S clusters [38]. Note that the absolute intensity of the signal in the cells expressing the C128S mutant is much lower than the signal of cells expressing the WT protein. The major spectral species (61%) has lower isomer shift and quadrupole splitting than expected for cubane Fe/S clusters, $\delta = 0.39 \text{ mm s}^{-1}$ and $\Delta E_Q = 0.89 \text{ mm s}^{-1}$. The values are close to what is known for $\gamma\text{-FeOOH}$ or similar oxidic species (isomer shift $\delta = 0.37 \text{ mm s}^{-1}$, $\Delta E_Q = 0.57 \text{ mm s}^{-1}$) from iron precipitations [39]. Oxidized 3Fe clusters, which might be considered as possible alternative explanation, although the isomer shift would be atypically high, can be excluded because of the absence of the sizable EPR signals from such clusters (see below). The second component in the Mössbauer spectrum again represents a non-Fe/S Fe(II) component: $\delta = 1.36 \text{ mm s}^{-1}$ and $\Delta E_Q = 2.86 \text{ mm s}^{-1}$. Thus, signals from Fe/S clusters cannot be detected in the Mössbauer spectrum of the mutated protein C128S.

Purified, reconstituted HCF101 showed a weak EPR signal at $g=2.02$ from a $[\text{3Fe-4S}]^{1+}$ cluster (Fig. 3b, top trace). The integrated intensity amounted to no more than 1% of the HCF101 molecules. Such a species could be derived from breakdown of a minor proportion of the $[\text{4Fe-4S}]^{2+}$ cluster, which could not be detected in the Mössbauer spectrum. Upon reduction with dithionite a broad rhombic EPR signal with g -values of 2.024 and 1.928 was observed (Fig. 3b, bottom trace), which was accompanied by a sharp isotropic $g=2.003$ radical signal, derived from dithionite breakdown products. The rhombic EPR signal had an integrated intensity of 0.05 spin/HCF101 in two different preparations. Since Mössbauer spectroscopy (Fig. 3a), iron and sulfide analysis and the extent of reduction as judged from visible spectroscopy (Fig. 2b) indicated the presence of a stoichiometric amount of [4Fe-

$4S]^{1+/2+}$ in HCF101 we were puzzled by the low intensity. Inspection of the low field region revealed a broad signal at $g=5$ from a $S=3/2$ form of the $[4Fe-4S]^{1+}$ cluster (Fig. 3b, bottom trace). This signal appeared upon reduction and was thus not from adventitiously bound high spin ferric ions, like the sharp $g=4.3$ signal in the top trace of Fig. 3c which disappeared upon reduction (Fig. 3c bottom trace). Further analysis of the $S=3/2$ signals was hampered by limitations imposed by solubility of the reconstituted HCF101 protein. It is likely that the $g=5$ signal encompasses the majority of the $[4Fe-4S]^{1+}$ clusters in HCF101, since upon double integration such weak signals represent more spins than sharp $S=1/2$ signals. Similar broad anisotropic $S=3/2$ signals have also been observed for the $[4Fe-4S]^{1+}$ cluster of the nitrogenase Fe-protein, [40] the activator of 2-hydroxyglutaryl-CoA dehydratase, [41] the Fx cluster in type I homodimeric photosynthetic reaction centre in heliobacteria, [42] and the Cfd1/Nbp35 complex (Netz, Pierik and Lill, unpublished). Since to our knowledge $S=3/2$ signals do not occur in natural $[2Fe-2S]^{1+}$ clusters, the presence of the $g=5$ signal in dithionite reduced HCF101 corroborates the presence of a $[4Fe-4S]^{1+/2+}$ cluster in HCF101 as shown by iron and sulfur analysis, UV-visible and Mössbauer spectroscopy.

HCF101 harbors an Fe/S cluster *in vivo* in *S. cerevisiae*

We attempted to obtain further *in vivo* evidence for the presence of an Fe/S cluster on HCF101. Since this is technically impossible in plant cells, we used baker's yeast and a ^{55}Fe radiolabeling assay. The coding sequence of mature, Myc-tagged HCF101 was cloned into a yeast expression vector containing or lacking a mitochondrial presequence (pre- $F_1\beta$). The plasmids were transformed into WT yeast cells and were able to produce significant amounts of protein in both mitochondria and cytosol (Fig. 4a, bottom panel). Cells were subjected to radiolabeling with ^{55}Fe , a cell extract was prepared and HCF101-Myc was affinity-purified using the Myc-tag. Strong radiolabeling of HCF101-Myc with ^{55}Fe was observed upon localization of the protein in mitochondria, but not in the cytosol (Fig. 4a, top). Since the latter result was not due to the lack of stability of HCF101 (Fig. 4a, bottom), our finding suggests that the yeast cytosol is not capable of forming the Fe/S cluster on HCF101. To analyze whether ^{55}Fe associated with mitochondria-targeted HCF101-Myc was indicating the binding of an Fe/S cluster, we analyzed the dependence of ^{55}Fe binding on the function of the cysteine desulfurase Nfs1, a central component of the mitochondrial ISC assembly system. When Nfs1 was depleted in Gal-NFS1 cells, which contain a regulatable *NFS1* gene, [6] a 75% decrease of ^{55}Fe incorporation was seen (Fig. 4b). This result shows that the ^{55}Fe incorporated into HCF101 is part of an Fe/S cluster. Taken together, the radiolabeling experiments in yeast document that HCF101 can bind an Fe/S cluster also in a eukaryotic system, yet it requires localization to mitochondria to achieve significant Fe/S cluster assembly on HCF101.

HCF101 can transfer its Fe/S cluster to apo-Leu1

A common feature of scaffold proteins is their capability to transfer the bound labile Fe/S cluster to the apoform of acceptor proteins [3,10,42]. We tested the putative scaffold function of HCF101 *in vitro*. It is at present not feasible to use apo-PSI complexes of higher plants to test the transfer Fe/S clusters from HCF101. We therefore chose yeast apo-Leu1 as a model acceptor protein. Upon binding of a $[4Fe-4S]$ cluster Leu1 exhibits isopropylmalate isomerase activity [32]. Apo-HCF101 was chemically reconstituted to its holoform (typically containing 3.6 ± 1.2 Fe and 3.0 ± 0.8 S per monomer) and mixed with apo-Leu1. Upon incubation of the two proteins under anaerobic conditions isopropylmalate isomerase enzyme activity developed fast (within 2 min) and efficiently (more than 90% conversion of apo-Leu1 to the active enzyme; Fig. 5). In contrast, upon chemical reconstitution of apo-Leu1 with similar amounts of iron and sulfide instead of a transferable Fe/S cluster, a rather slow and inefficient generation of the Leu1 enzyme activity was observed. Even after 30 min only about one third

of the activity was detected as compared to that using HCF101 as a donor after 2 min. These data show that the HCF101-bound Fe/S cluster is labile and can be efficiently transferred to acceptor proteins. Hence, HCF101 fulfills a critical requirement for a scaffold function, i.e. the capacity to transfer a labile Fe/S cluster to a target protein under conditions when chemical reconstitution is inefficient.

DISCUSSION

In the present study we biochemically investigated the molecular function of the plastid representative HCF101 of the FSC superfamily of P-loop NTPases. The protein was previously shown in cell biological experiments to function as an essential factor for assembly of PSI but the mechanism of action remained unknown. Levels of PSI and FTR complexes harboring three and one [4Fe-4S] clusters, respectively, were significantly diminished in the *hcf101* mutant although translation of PSI proteins was not affected [23]. Bacterial, cytosolic, and mitochondrial members of the FSC family have been described to bind [4Fe-4S] clusters and their role in Fe/S cluster biogenesis in their particular cell compartments has been documented [26,31,43,44]. However, despite the overall striking conservation of the protein signatures, surprisingly the typical and conserved Fe/S cluster binding motifs of all other FSC members are absent in HCF101 (Fig. 1) [23]. Therefore it was still a matter of debate whether HCF101 has the ability to bind and to transfer an Fe/S cluster or if it possibly acquired other functions in the course of evolution. Our results show that heterologously expressed HCF101 in *E. coli* retained approximately 1 Fe and 1 S per HCF101 monomer after purification, and that after chemical reconstitution about 4 Fe and 4 S per HCF101 monomer were present. This is consistent with the binding of a [4Fe-4S] cluster. Indeed, UV-visible, Mössbauer and EPR spectroscopy of reconstituted HCF101 confirmed the presence of a [4Fe-4S] cluster. Interestingly, the reduced species of HCF101 is mainly present in the S=3/2 form. It is remarkable that this type of EPR signal has been encountered in many proteins which have solvent-exposed clusters. The property of HCF101 to rapidly transfer its cluster to apo-Leu1 suggests that its cluster should also be solvent-exposed rather than buried in the protein. Taken together, in this study we unequivocally demonstrate by several methodologies, that despite the lack of FSC-family characteristic binding motifs, HCF101 harbors a labile [4Fe-4S] cluster.

In vitro analyses using site-directed mutagenesis in *E. coli* identified three cysteine residues that are essential for binding the Fe/S cluster. As expected the only cysteine which is also conserved in cyanobacteria, C128, is most crucial for the assembly/stability of the cluster when expressed in *E. coli*. The other two cysteine residues involved in cluster binding, C347 and C419, are present in the context of a second cysteine, namely as part of a CX₇C and a CX₄C motif, respectively. These motifs might resemble metal binding sites. The other two cysteine residues of these motifs are also likely to be involved in Fe/S cluster binding and/or stabilization as the cluster of the respective purified mutant forms were more unstable as compared to the WT protein (data not shown). In contrast to all other FSC members a cysteine residue is present in the highly conserved P-loop domain of the plant HCF101 (CKGGVVGKS). This might indicate a change of function of this domain in the plant lineage, although the newly evolved cysteine in the P-loop is not essentially required for Fe/S cluster binding (not shown). Two Fe/S cluster binding cysteines (C97 and C131) have been identified in the homodimeric protochlorophyllide oxidoreductase subunit BchL, a NifH-like protein sharing some sequence similarity with HCF101 [45]. One of the BchL cysteines (C131) is conserved in HCF101 (C303), yet might have lost its function as a cluster ligand.

Nevertheless, with the exception of C128, which is also conserved in cyanobacteria, all other cysteine residues are not preserved in other organisms (Supplementary Fig. 1) pointing to a specialized plastid form of class I FSC proteins. Therefore, HCF101 may represent a

divergent type of Fe/S cluster binding protein, which presumably evolved as an adaptation to oxygen evolution in the chloroplast. So far almost nothing is known about the formation of [4Fe-4S] clusters in plastids and their insertion into PSI and other chloroplast complexes. To clarify the possible function of HCF101 regarding postranslational assembly of PSI, the *in vitro* cluster transfer properties of the [4Fe-4S] cluster on HCF101 was tested. Since it is not feasible to establish an *in vitro* assay for the transfer of [4Fe-4S] clusters on the apo-PSI complex or to study Fe/S cluster transfer in a living plant cell, we used the yeast apo-Leu1 as a model target protein. HCF101 efficiently transferred its cluster to apo-Leu1 demonstrating that it binds its [4Fe-4S] cluster in a labile fashion. These data suggest that HCF101 could transfer its Fe/S cluster to apo-proteins in chloroplasts. Whether the HCF101 function is specific for [4Fe-4S] clusters, cannot be derived from *in vitro* experiments, since one has to recognize the limitations of *in vitro* Fe/S transfer. Nevertheless, it is generally accepted that the ability to rapidly and efficiently transfer Fe/S cluster (s) *in vitro* to acceptor apo-proteins is a *sine qua non* requisite for scaffold function of a Fe/S cluster-containing protein. As an alternative scaffold protein NFU2 which binds [2Fe-2S] clusters has been described in chloroplasts [18,19]. Somewhat surprisingly, *Arabidopsis nfu2* mutants also have a PSI defect, even though these plants exhibit a much milder phenotype than *hcf101* plants. At present, it is unknown whether NFU2 can assemble [4Fe-4S] clusters by itself, or transfer the assembled [2Fe-2S] clusters onto HCF101, thus forming transient [4Fe-4S] clusters.

Our biochemical studies suggest that HCF101 can assemble a [4Fe-4S] cluster and transfer it to apoproteins. It is thus conceivable that this protein fulfils a similar function as the Cfd1-Nbp35 complex performing a scaffold role in cytosolic and nuclear Fe/S protein assembly, or mitochondrial Ind1 involved in Fe/S cluster incorporation into respiratory complex I. Strikingly, the motifs transiently binding the Fe/S clusters in these latter proteins differ markedly from HCF101. Future studies therefore will have to uncover the precise mode of how the Fe/S cluster is bound to HCF101. Moreover, it has to be addressed whether Fe/S cluster assembly on HCF101 occurs spontaneously in the plastid, i.e. HCF101 serves as a true scaffold, or whether Fe/S cluster assembly depends on other scaffolds such as NFU1, NFU2 and NFU3, or the SufA-type scaffolds [46], and has some specificity for delivering the Fe/S cluster to [4Fe-4S] proteins.

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REFERENCES

1. Beinert, H. (2000) Iron-sulfur proteins: ancient structures, still full of surprises. *J. Biol. Inorg. Chem.* **5**, 2-15
2. Frazzon, J. and Dean, D. R. (2003) Formation of iron-sulfur clusters in bacteria: an emerging field in bioinorganic chemistry. *Curr. Opin. Chem. Biol.* **2**, 166-173
3. Johnson, D. C., Dean, D. R., Smith, A. D. and Johnson M. K. (2005) Structure, function, and formation of biological iron-sulfur clusters. *Annu. Rev. Biochem.* **74**, 247-281
4. Zheng, L., White, R. H., Cash, V. L., Jack, R. F. and Dean, D. R. (1993) Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. *Proc. Natl. Acad. Sci. U S A* **90**, 2754-2758
5. Zheng, L., Cash, V.L., Flint, D.H. and Dean, D.R. (1998) *J. Biol. Chem.* **273**, 13264-13272
6. Kispal, G., Csere, P., Prohl, C. and Lill R. (1999) The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. *EMBO J.* **18**, 3981-3989
7. Takahashi, Y. and Tokumoto, U. (2002) A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. *J. Biol. Chem.* **277**, 28380-28383
8. Jacobson, M. R., Brigle, K. E., Bennett, L. T., Setterquist, R. A., Wilson, M. S., Cash, V. L., Beynon, J., Newton, W. E. and Dean, D. R. (1989) Physical and genetic map of the major *nif* gene cluster from *Azotobacter vinelandii*. *Mol. Gen. Genet.* **171**, 1017-1027
9. Lill, R. and Mühlenhoff, U. (2005) Iron-sulfur-protein biogenesis in eukaryotes. *Trends Biochem. Sci.* **3**, 133-141
10. Lill R. and Mühlenhoff U. (2008) Maturation of Iron-Sulfur Proteins in Eukaryotes. Mechanisms, Connected Processes, and Diseases. *Annu. Rev. Biochem.* **77**, 669-700
11. Amann, K., Lezhneva, L., Wanner, G., Herrmann, R. G. and Meurer J. (2004) Accumulation of Photosystem one 1, a member of a novel gene family, is required for accumulation of [4Fe-4S] cluster-containing chloroplast complexes and antenna proteins. *Plant Cell* **16**, 3084-3097
12. Balk, J. and Lobréaux. S. (2005) Biogenesis of iron-sulfur proteins in plants. *Trends Plant Sci.* **7**, 324-331
13. Kessler, D. and Papenbrock, J. (2005) Iron-sulfur cluster biosynthesis in photosynthetic organisms. *Photosynth. Res.* **86**, 391-407
14. Pilon, M., Abdel-Ghany, S. E., Van Hoewyk, D., Ye H. and Pilon-Smits, E. A. (2006) Biogenesis of iron-sulfur cluster proteins in plastids. *Genet. Eng. (N Y)* **27**, 101-117
15. Ye, H., Garifullina, G. F., Abdel-Ghany, S. E., Zhang, L., Pilon-Smits, E. A. and Pilon M. (2005) *Planta* **220**, 602-608
16. Van Hoewyk, D., Abdel-Ghany, S. E., Cohu, C. M., Herbert, S. K., Kugrens, P., Pilon, M. and Pilon-Smits, E. A. (2007) Chloroplast iron-sulfur cluster protein maturation requires the essential cysteine desulfurase CpNifS. *Proc. Natl. Acad. Sci. USA* **104**, 5686-5691
17. Ye, H., Abdel-Ghany, S. E., Anderson, T. D., Pilon-Smits, E. A. and Pilon, M. (2006) The chloroplast NifS-like protein of *Arabidopsis thaliana* is required for iron-sulfur cluster formation in ferredoxin. *J. Biol. Chem.* **281**, 8958-8969
18. Yabe, T., Morimoto, K., Kikuchi, S., Nishio, K., Terashima, I. and Nakai, M. (2004) The *Arabidopsis* chloroplastic NifU-like protein CnfU, which can act as an iron-sulfur cluster scaffold protein, is required for biogenesis of ferredoxin and photosystem I. *Plant Cell* **16**, 993-1007
19. Touraine, B., Boutin, J. P., Marion-Poll, A., Briat, J. F., Peltier, G. and Lobréaux, S. (2004) Nfu2: a scaffold protein required for [4Fe-4S] and ferredoxin iron-sulphur cluster assembly in *Arabidopsis* chloroplasts. *Plant J.* **40**, 101-111

20. Abdel-Ghany, SE, Ye, H, Garifullina, GF, Zhang, L, Pilon-Smits, EA and Pilon, M. (2005), Iron-sulfur cluster biogenesis in chloroplasts. Involvement of the scaffold protein CpIscA. *Plant Physiol.* **138**, 161-172
21. Bandyopadhyay, S., Gama, F., Molina-Navarro, M. M., Gualberto, J. M., Claxton, R., Naik, S. G., Huynh, B. H., Herrero, E., Jacquot, J. P., Johnson, M. K. and Rouhler, N. (2008) Chloroplast monothiol glutaredoxins as scaffold proteins for the assembly and delivery of [2Fe-2S] clusters. *EMBO J.* **27**, 1122-1133
22. Wollenberg, M., Berndt, C., Bill, E., Schwenn, J. D. and Seidler, A. (2003) A dimer of the FeS cluster biosynthesis protein IscA from cyanobacteria binds a [2Fe2S] cluster between two protomers and transfers it to [2Fe2S] and [4Fe4S] apo proteins. *Eur. J. Biochem.* **270**, 1662-1671
23. Lezhneva, L., Amann, K. and Meurer, J. (2004) The universally conserved HCF101 protein is involved in assembly of [4Fe-4S]-cluster-containing complexes in *Arabidopsis thaliana* chloroplasts. *Plant J.* **37**, 174-185
24. Stöckel, J. and Oelmüller, R. (2004) A novel protein for photosystem I biogenesis. *J. Biol. Chem.* **279**, 10243-10251
25. Skovran, E. and Downs, D. M. (2003) Lack of the ApbC or ApbE protein results in a defect in [Fe-S] cluster metabolism in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **185**, 98-106
26. Boyd, J. M., Pierik, A. J., Netz, D. J., Lill, R. and Downs D. M. (2008) Bacterial ApbC Can Bind and Effectively Transfer Iron-Sulfur Clusters. *Biochemistry* **47**, 8195-8202
27. Boyd, J. M., Sondelski, J. L. and Downs DM. (2009) Bacterial ApbC protein has two biochemical activities that are required for *in vivo* function. *J. Biol. Chem.* **284**, 110-118
28. Bych, K., Kerscher, S., Netz, D. J., Pierik A. J., Zwicker K., Huynen M. A., Lill R., Brandt U. and Balk J. (2008) The iron-sulphur protein Ind1 is required for effective complex I assembly. *EMBO J.* **27**, 1736-1746
29. Bych K., Netz D. J., Vigani G., Bill E, Lill R., Pierik A. J., Balk J. (2008) The essential cytosolic iron-sulfur protein Nbp35 acts without Cfd1 partner in the green lineage. *J Biol Chem.* **283**, 35797-35804
30. Roy, A., Solodovnikova, N., Nicholson, T., Antholine, W. and Walden, W. E. (2003) A novel eukaryotic factor for cytosolic Fe-S cluster assembly. *EMBO J.* **22**, 4826-4835
31. Hausmann, A., Aguilar Netz, D. J., Balk, J., Pierik, A. J., Mühlhoff U. and Lill, R. (2005) The eukaryotic P-loop NTPase Nbp35: an essential component of the cytosolic and nuclear iron-sulfur protein assembly machinery. *Proc. Natl. Acad. Sci. USA* **102**, 3266-3271
32. Netz, D. J., Pierik, A. J., Stümpfig, M., Mühlhoff, U. and Lill R. (2007) The Cfd1-Nbp35 complex acts as a scaffold for iron-sulfur protein assembly in the yeast cytosol. *Nat. Chem. Biol.* **3**, 278-286
33. Meurer, J., Plücker, H., Kowallik, K. V. and Westhoff P. (1998) A nuclear-encoded protein of prokaryotic origin is essential for the stability of photosystem II in *Arabidopsis thaliana*. *EMBO J.* **17**, 5286-5297
34. Matzanke, B. F. (1994) in *Transition Metals in Microbial Metabolism* (Winkelmann, G., and Carrano, C. J., eds) pp. 117-158, Harwood Academic Publishers GmbH, Amsterdam
35. Böhnke, R. and Matzanke, B. F. (1995) The mobile ferrous iron pool in *Escherichia coli* is bound to a phosphorylated sugar derivative. *BioMetals* **8**, 223-230
36. Bensadoun, A. and Weinstein, D. (1976) Assay of proteins in the presence of interfering materials. *Anal. Biochem.* **70**, 241-250
37. Pierik, J., Wolbert, R., Mutsaers, P., Hagen, W. and Veeger, C. (1992) Purification and biochemical characterization of a putative [6Fe-6S] prismatic-cluster-containing protein from *Desulfovibrio vulgaris* (Hildenborough) *Eur. J. Biochem.* **206**, 697-704

38. Schünemann, V. and Winkler, H. (2000) Structure and dynamics of biomolecules studied by Mössbauer spectroscopy. *Reports on Progress in Physics* **63**, 263-353
39. Greenwood, N. and Gibb, T. C. (1971) *Mössbauer Spectroscopy*. Chapman and Hall Ltd: London
40. Hagen, W. R., Eady, R. R., Dunham, W. R. and Haaker H. (1985) A novel $S = 3/2$ EPR signal associated with native Fe-proteins of nitrogenase. *FEBS Lett.* **189**, 250-254
41. Hans, M., Buckel, W. and Bill, E. (2000) The iron-sulfur clusters in 2-hydroxyglutaryl-CoA dehydratase from *Acidaminococcus fermentans*. Biochemical and spectroscopic investigations. *Eur. J. Biochem.* **267**, 7082-7093
42. Lill, R. and Mühlhoff, U. (2006) Iron-sulfur protein biogenesis in eukaryotes: components and mechanisms. *Annu. Rev. Cell Dev. Biol.* **22**, 457-486
43. Stehling, O., Netz, D. J., Niggemeyer, B., Rösser, R., Eisenstein, R. S., Puccio, H., Pierik, A. J. and Lill, R. (2008) The human CIA component huNbp35 is essential for both cytosolic iron-sulfur protein assembly and iron homeostasis. *Mol. Cell. Biol.* **28**, 5517-5528
44. Kohbushi, H., Nakai, Y., Kikuchi, S., Yabe, T., Hori, H. and Nakai M. (2009) Arabidopsis cytosolic Nbp35 homodimer can assemble both [2Fe-2S] and [4Fe-4S] clusters in two distinct domains. *Biochem. Biophys. Res. Commun.* **378**, 810-815
45. Bröcker, M. J., Virus, S., Ganskow, S., Heathcote, P., Heinz, D. W., Schubert, W. D., Jahn, D. and Moser, J. (2008) ATP-driven reduction by dark-operative protochlorophyllide oxidoreductase from *Chlorobium tepidum* mechanistically resembles nitrogenase catalysis. *J. Biol. Chem.* **283**, 10559-10567
46. Xu XM, Møller SG. (2008) Iron-sulfur cluster biogenesis systems and their crosstalk. *Chembiochem.* **9**, 2355-2362

Figure Legends

Figure 1 Schematic alignment of cysteine residues in members of the FSC superfamily of P-loop NTPases in eukaryotes and eubacteria

The plastid and mitochondrial forms (classes I and II, respectively) in *Arabidopsis*, the cytosolic class III and IV homologues (Nbp35 and Cfd1) in yeast and the eubacterial homologue ApbC (class 1) in *Salmonella enterica* are represented. Cysteines indicated with dotted lines are highly conserved within the four classes, but none of those cysteines is present in the chloroplast HCF101 protein. TPM and TPc indicate mitochondrial and chloroplast transit peptides, respectively.

Figure 2 Purified HCF101 binds one [4Fe-4S] cluster per monomer *in vitro*

(a) SDS PAGE and Coomassie staining of *E. coli* extracts and purified HCF101 proteins were performed. Separation of an *E. coli* extract containing overproduced HCF101 analyzed 5 h after induction (lane 3) An extract from cells containing the empty pET23a⁺ vector is shown as a control (lane 2). Lanes 4-12 show the purified wild-type (WT) and the indicated eight recombinant cysteine mutant HCF101 proteins after anion exchange chromatography. Lane 1 contains molecular mass standards. (b) UV-visible absorption spectra of WT HCF101 were performed. They show a distinct shoulder at 420 nm in the aerobically isolated sample and after the chemical reconstitution under anaerobic conditions. The peak disappeared after reduction with 1 mM sodium dithionite (DTN). (c) The Fe and S content of the purified HCF101 protein was determined before and after chemical reconstitution for six independent preparations. The iron content of 1.1 ± 0.3 (as isolated) and 3.6 ± 1.2 (reconstituted) and the sulfur content of 0.6 ± 0.2 (as isolated) and 3.0 ± 0.8 (reconstituted) suggest binding of one

[4Fe-4S] cluster per monomer after reconstitution. **(d)** Cysteine residues of HCF101 required for stable Fe/S cluster binding *in vitro* were identified. UV-visible absorption spectra of purified WT and mutated HCF101 proteins (C128S, C347S, C419S), lacking the shoulder at 420 nm. A protein concentration of 1 mg/ml was used in **(b)** and **(d)**.

Figure 3 Spectroscopic analysis of HCF101

(a) Mössbauer spectra of intact *E. coli* cells were performed after overexpression of HCF101 WT (upper panel) and HCF101 C128S (lower panel) in ^{57}Fe enriched medium. Equal expression of protein was checked by SDS PAGE. **(b)** Chemically reconstituted HCF101 was analyzed by EPR spectra before (top trace) and after reduction (bottom trace) with 2 mM sodium dithionite. EPR conditions: temperature 10 K; microwave frequency, 9420 ± 1 MHz; modulation amplitude, 1.25 mT; modulation frequency, 100 kHz; microwave power 1.26 mW. **(c)** As in **(b)**, but at 20 mW microwave power.

Figure 4 HCF101 can assemble an Fe/S cluster in yeast mitochondria

(a) W303 WT cells were transformed with vectors pRS426-TDH3 encoding a Myc-tagged HCF101 with (Mito) or without (Cyto) an N-terminal F_1F_0 -ATPase mitochondrial targeting sequence or with the empty vector. After growth in minimal medium with 2% (w/v) glucose lacking iron for 40 h, cells were radiolabeled with ^{55}Fe and incubated for 2 h. Cell lysates were prepared and HCF101 was affinity-purified using anti-Myc beads. The amount of ^{55}Fe bound to HCF101-Myc was measured by scintillation counting. **(b)** Gal-NFS1 cells in which the *NFS1* gene is under the control of a *GAL1-10* promoter were transformed with pRS426-TDH3 encoding the mitochondria-targeted Myc-tagged HCF101. After growth in 2% (w/v) galactose- (Gal) or 2% (w/v) glucose-containing (Glc) iron-poor minimal medium for 40 h, ^{55}Fe radiolabeling, preparation of cell lysates and analysis of ^{55}Fe binding were carried out as described above. The bottom panels are immunostains of TCA-precipitated cell lysates separated on 12.5 % SDS-PAGE and probed with the indicated antibodies.

Figure 5 Holo-HCF101 mediated transfer of the [4Fe-4S] cluster to the apo-Leu1 target protein

Reconstituted and desalted holo-HCF101 (3.85 μM) was mixed with reduced apo-Leu1 (2.45 μM) at 23°C in buffer A. Isopropylmalate isomerase activity was measured at the indicated time points (circles). In a subsequent control experiment, activation of 2.45 μM apo-Leu1 with ferric ammonium citrate and Li_2S at concentrations identical to those present in HCF101 (15.4 μM each) was assayed for isopropylmalate isomerase activity (triangles).

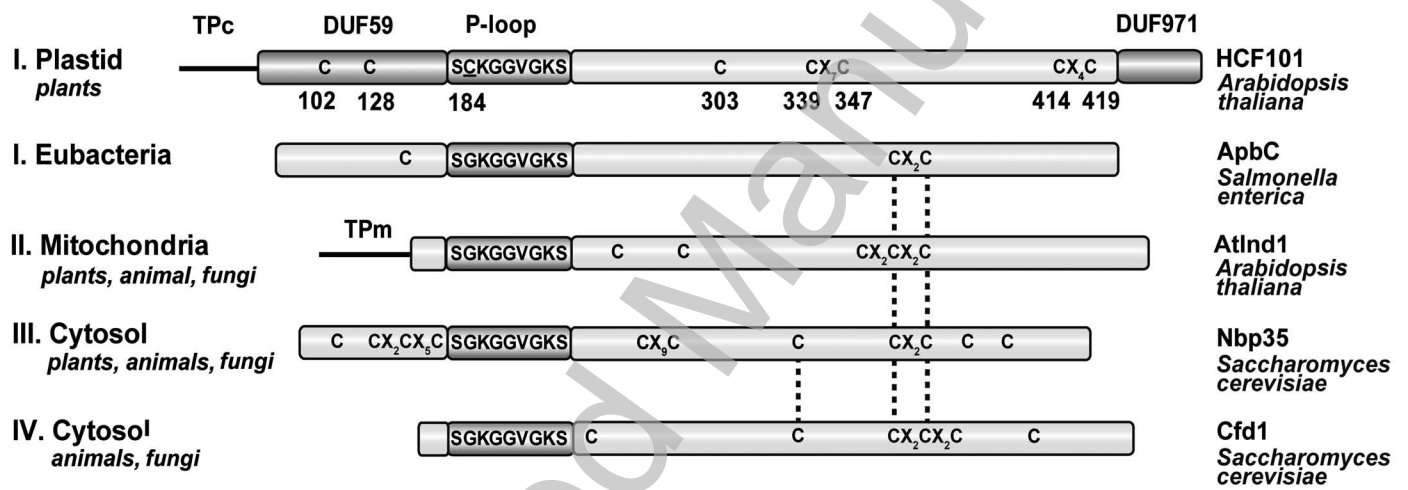


Figure 1

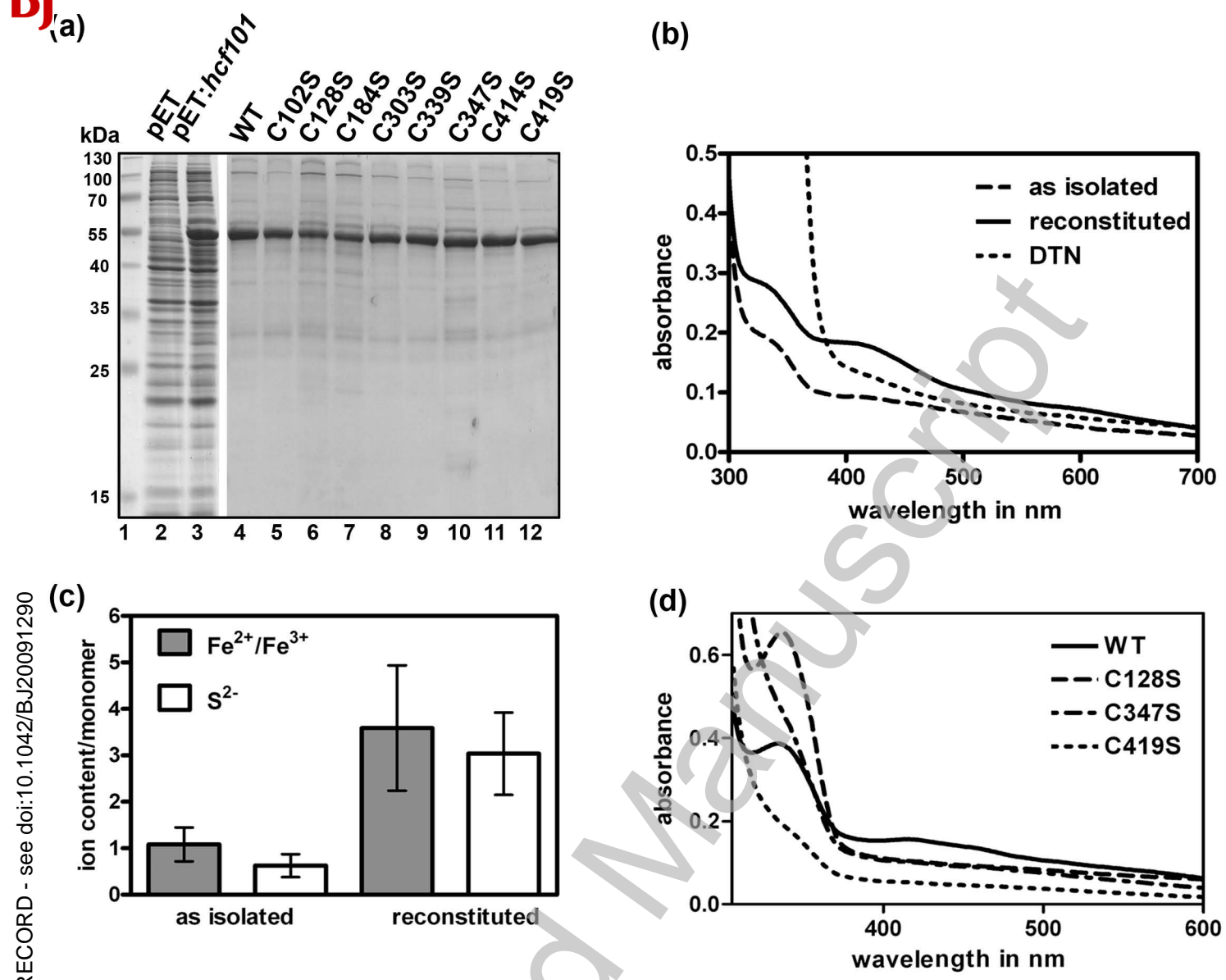


Figure 2

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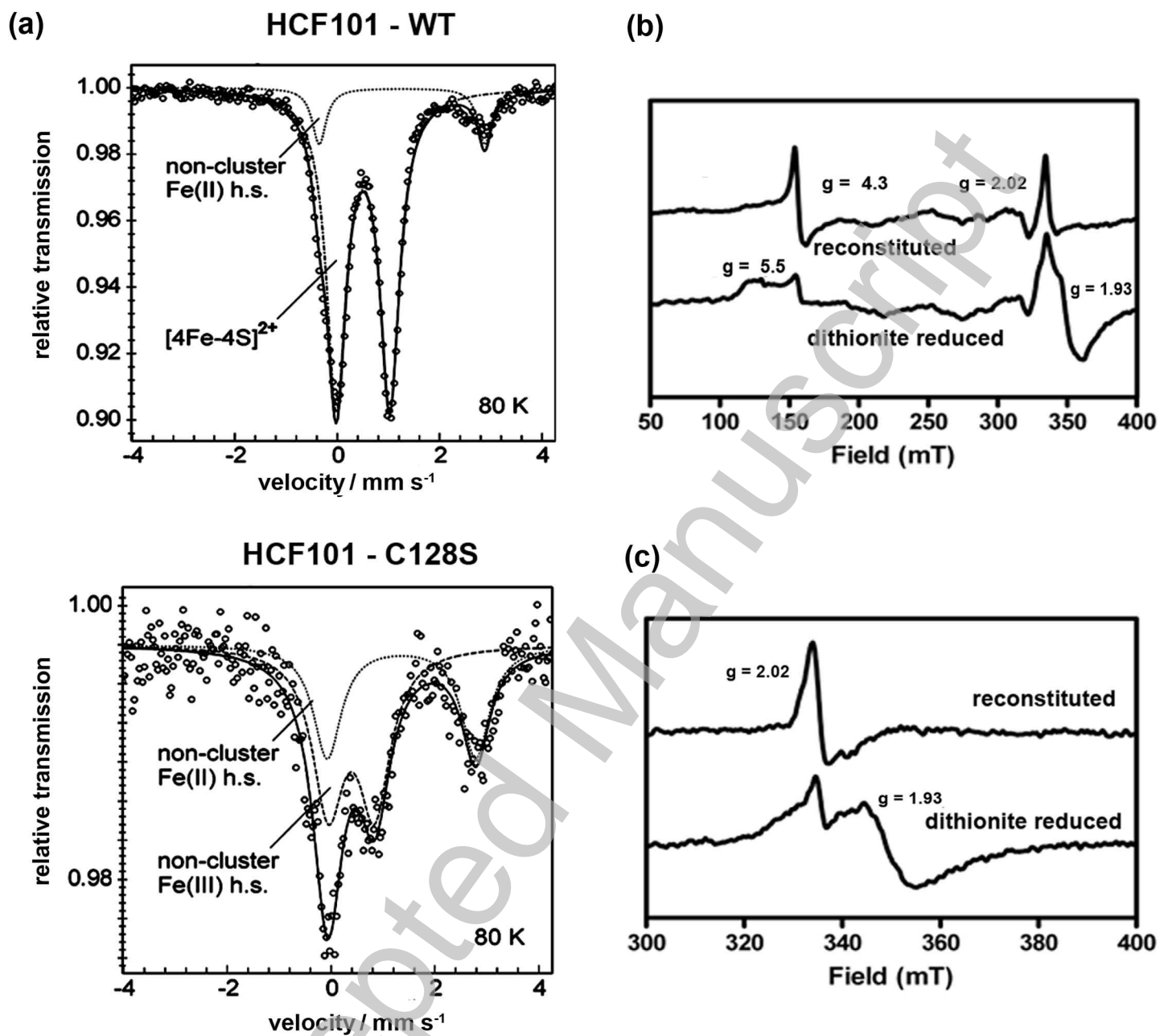


Figure 3

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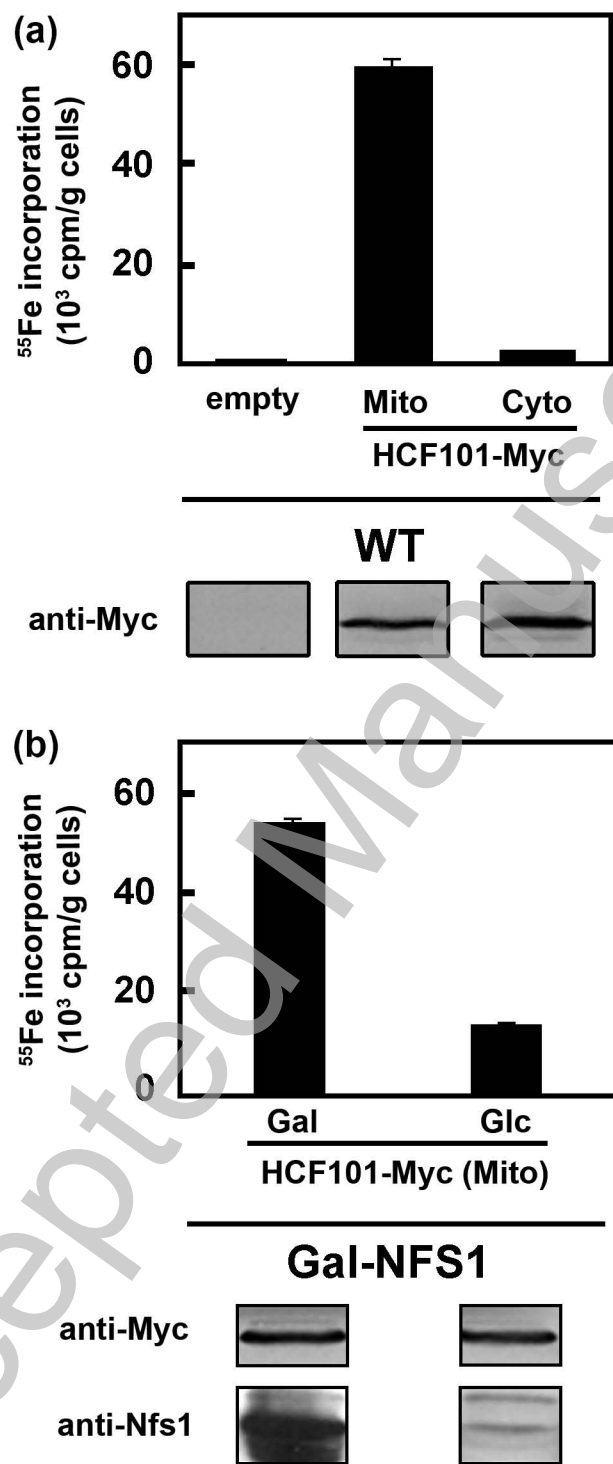


Figure 4

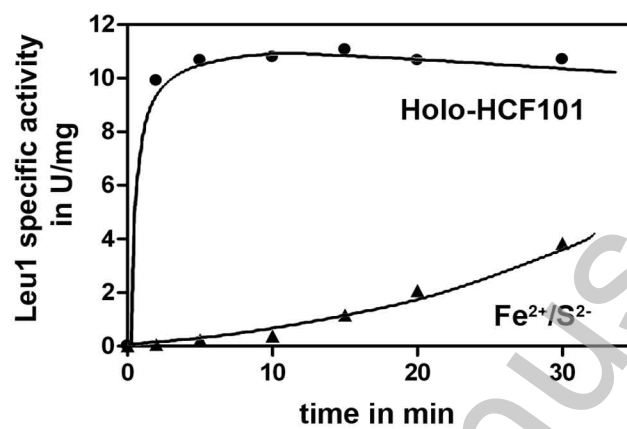


Figure 5