Mitochondrion 12 (2012) 539–549

Contents lists available at SciVerse ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Identification of a Nfs1p-bound persulfide intermediate in Fe–S cluster synthesis by intact mitochondria

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ARTICLE INFO

Article history:
Received 7 May 2012
Received in revised form 9 July 2012
Accepted 10 July 2012
Available online 17 July 2012

Keywords:
Cysteine desulfurase
Nfs1p•Isd11p complex
Persulfide
Fe–S clusters
Aconitase
Yeast

ABSTRACT

Cysteine desulfurases generate a covalent persulfide intermediate from cysteine, and this activated form of sulfur is essential for the synthesis of iron–sulfur (Fe–S) clusters. In yeast mitochondria, there is a complete machinery for Fe–S cluster synthesis, including a cysteine desulfurase, Nfs1p. Here we show that following supplementation of isolated mitochondria with [^{35}S]cysteine, a radiolabeled persulfide could be detected on Nfs1p. The persulfide persisted under conditions that did not permit Fe–S cluster formation, such as nucleotide and/or iron depletion of mitochondria. By contrast, under permissive conditions, the radiolabeled Nfs1p persulfide was greatly reduced and radiolabeled aconitase was formed, indicating transfer of persulfide to downstream Fe–S cluster recipients. Nfs1p in mitochondria was found to be relatively more resistant to inactivation by N-ethylmaleimide (NEM) as compared with a prokaryotic cysteine desulfurase. Mitochondria treated with NEM (1 mM) formed the persulfide on Nfs1p but failed to generate Fe–S clusters on aconitase, likely due to inactivation of downstream recipient(s) of the Nfs1p persulfide. Thus the Nfs1p-bound persulfide as described here represents a precursor *en route* to Fe–S cluster synthesis in mitochondria.
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1. Introduction

Iron–sulfur (Fe–S) clusters consist of iron complexed with inorganic sulfur in protein-bound structures capable of varied biochemical reactions. In cells, these clusters are essential cofactors in proteins that mediate diverse functions including electron transfer, oxygen interaction, catalysis, iron regulation, DNA repair, ribosome biogenesis, and tRNA modification among others (Johnson et al., 2005; Lill, 2009; Rouault and Tong, 2008). Fe–S cluster biogenesis is an evolutionarily conserved multi-step biochemical process involving more than a dozen protein components. The process can be broadly understood in terms of three phases (Stemmler et al., 2010). In the first phase, sulfur is mobilized from cysteine by an enzyme cysteine desulfurase. In the second phase, sulfur is transferred from the enzyme to scaffold proteins and assembled with iron to form an Fe–S cluster intermediate. In the third phase, mediated by chaperones and glutaredoxins, the Fe–S cluster intermediate is transferred to apoproteins, forming holo and active proteins. In all known cases, the sulfur for Fe–S cluster assembly is derived from cysteine via the action of cysteine desulfurases. The biochemical mechanism of these enzymes has been defined by the pioneering work of Dean and co-workers on the NifS ortholog from the nitrogen-fixing prokaryote *Azotobacter vinelandii* (Zheng et al., 1993, 1994).

Briefly, a conserved lysine forms a Schiff base with the pyridoxal phosphate (PLP) cofactor. The enzyme substrate cysteine interacts with the cofactor, creating a cysteine–PLP ketimine adduct. Nucleophilic attack by the thiolate anion of the active site cysteine on the sulfhydryl group of the substrate–PLP adduct cleaves the C–S bond, resulting in formation of an enzyme-bound persulfide (Enz–S–SH). The substrate cysteine in the PLP adduct is converted to alanine and subsequently released, thereby regenerating PLP for a new catalytic cycle (Johnson et al., 2005; Mihara and Esaki, 2002; Selbach et al., 2010; Zheng et al., 1994). As needed, sulfane sulfur of the persulfide is transferred from the enzyme active site to specific recipients such as Isu type scaffolds involved in Fe–S cluster assembly. After sulfur transfer, the active site cysteine and the substrate-binding site of the cysteine desulfurase enzyme are restored to their original state, thus facilitating another cycle of sulfur supply.

In *Saccharomyces cerevisiae*, the cysteine desulfurase is encoded by a single gene *NFS1*. The Nfs1p protein is found mostly in mitochondria (Li et al., 1999), with a small amount functioning in extramitochondrial locations (Nakai et al., 2001). Nfs1p is required for activities of Fe–S cluster proteins (Kispal et al., 1999; Li et al., 1999). Point mutations in the conserved lysine for PLP attachment or in the conserved active site cysteine lead to cell inviability, suggesting that Nfs1p-bound persulfide formation is essential for Fe–S cluster synthesis in mitochondria (Li et al., 1999). In agreement with this notion, a hypomorphic *nfs1* mutant exhibits high cellular iron uptake and accumulates iron in mitochondria. These phenotypic defects are similar to those observed in other yeast mutants that are

Abbreviations: PLP, pyridoxal phosphate; NEM, N-ethylmaleimide.

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doi:10.1016/j.mito.2012.07.103

deficient in mitochondrial Fe–S clusters (Li et al., 1999). Interestingly, some *nfs1* mutants can suppress the lysine auxotrophy of cells lacking the Cu/Zn superoxide dismutase (Strain et al., 1998). Furthermore, Nfs1p appears to be required for thiolation of certain tRNAs involved in mitochondrial protein synthesis (Nakai et al., 2004). Nfs1p associates with a small mitochondrial protein, Isd11p (~11 kDa), and forms a complex (Nfs1p•Isd11p) of ~200 kDa, but the function of Isd11p in mitochondria has not been determined (Adam et al., 2006; Wiedemann et al., 2006). Like the yeast Nfs1p, the major portion of human Nfs1 is also found in mitochondria with a small amount in cytosol/nucleus, and knockdown of the human enzyme by siRNA is lethal likely due to severe deficiency of Fe–S clusters in both mitochondria and cytoplasm (Biederbick et al., 2006; Fosset et al., 2006). Furthermore, the human enzyme also interacts with the homologous Isd11 protein (Shi et al., 2009). However, identification of a bona fide persulfide intermediate on a eukaryotic cysteine desulfurase that can be productively used for Fe–S cluster synthesis in intact mitochondria thus far remained elusive. Here we have resolved this important issue.

Specifically, we present the results of studies using isolated and intact yeast mitochondria supplemented with [^{35}S]cysteine. The [^{35}S] label detected on Nfs1p reflects the cysteine desulfurase activity of the enzyme in mitochondria. Another novel feature of these assays performed with intact mitochondria is that the sulfur label can be detected as newly formed Fe–^{35}S clusters on aconitase in a nucleotide and iron dependent manner (Amutha et al., 2008, 2009; Pain et al., 2010). Here we show that the signal on Nfs1p reflecting persulfide formation and the signal on aconitase reflecting new Fe–S cluster synthesis vary reciprocally, indicating a precursor–product relationship.

2. Materials and methods

2.1. Yeast strains, plasmids, growth conditions, and mitochondria isolation

Regulated expression of Nfs1p from the *GAL10* promoter was accomplished by use of the pEMBlyex4i vector carrying the *NFS1* open reading frame (ORF) and integrated at the *URA3* locus of a Δ*nfs1::HIS3* yeast strain, as previously described (Li et al., 1999). The Gal–Nfs1p strain was grown at 30 °C to mid-logarithmic phase under inducing conditions in YPAR galactose (1% yeast extract, 2% peptone, 100 μg/ml adenine, 2% raffinose and 0.2% galactose). Cells were harvested, resuspended in YPAR with or without galactose (0.2%), allowed to grow at 30 °C for 22 h, and mitochondria were isolated as described (Amutha et al., 2009). Nfs1p persulfide formation or Fe–S cluster biogenesis in mitochondria, isolated from the Gal–Nfs1p strain with the Nfs1p protein expressed, was very similar to those in mitochondria isolated from wild-type strains (D273-10B, BY4741 or YPH499). D273-10B mitochondria were used as wild-type controls. The deletion mutant for *GGC1* carrying the Δ*ggc1*::KanMX marker was constructed as described (Gordon et al., 2006; Lesuisse et al., 2004).

2.2. Bacterial expression of proteins

In *S. cerevisiae*, the precursor form of the mitochondrial Nfs1p contains 497 amino acids. Upon import into mitochondria, the N-terminal targeting signal (36 amino acids) of the Nfs1p precursor protein is removed by two different proteases, generating the mature form of the protein (461 amino acids) (Naamati et al., 2009). Unlike in the case for Nfs1p, the accessory protein Isd11p does not appear to contain a cleavable mitochondrial targeting signal (Adam et al., 2006; Wiedemann et al., 2006). The plasmids pST39 and pET3aTr were obtained from Dr. Song Tan (Tan, 2001), and a construct (plasmid 20–38) was generated that contained a T7 promoter driving a polycistronic mRNA for mature Nfs1p with a C-terminal His_6 tag (Nfs1p–His_6) and Isd11p, each with separate ribosome-binding sites. For expression of *A. vinelandii* NifS, the plasmid pT7-7/NifS (pDB551) was obtained from Dr. Dennis R. Dean (Zheng et al., 1993). The ORF was modified with a 5′ NdeI restriction site and a 3′ His_6 tag followed by a stop codon and BamHI site, generating the plasmid, pT7-7/NifS–His_6.

Proteins were expressed in BL21 (DE3) Codon Plus cells (Stratagene). Cells carrying different plasmids were grown in LB media containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol to OD_{600} of ~0.6. Conditions for expression of proteins were optimized so that the majority of each protein was in soluble form (Amutha and Pain, 2003; Pain et al., 2010). Co-expression of Nfs1p–His_6 and Isd11p was carried out in the presence of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 24 h at 20 °C. Expression of *A. vinelandii* NifS–His_6 was induced with 0.5 mM IPTG for 3 h at 37 °C. Cells were harvested, and washed with buffer A (50 mM Tris/HCl, pH 8.0, 0.15 M NaCl, 10% glycerol, 1 mM PMSF). Cells were resuspended in buffer A, incubated with lysozyme (50 μg/ml) for 30 min on ice, and then disrupted using a probe sonicator (Branson Sonifier 450). Cell lysates were centrifuged at 12,000 × *g* for 30 min at 4 °C, and the supernatant fraction was incubated with Ni-NTA agarose by end-over-end mixing for 3 h at 4 °C. The resin was washed with buffer A containing 10 mM imidazole, and bound proteins were eluted with 0.4 M imidazole in buffer A. Purified proteins were stored at −80 °C until further use.

2.3. Cysteine desulfurase assays

As necessary, experiments were performed with or without prior depletion of mitochondrial nucleotides. To deplete endogenous nucleotides, isolated and intact mitochondria were preincubated at 30 °C for 10 min (Amutha et al., 2008, 2009; Pain et al., 2010). In a typical 50 μl assay, mitochondria (200 μg of proteins) in HS buffer (20 mM Hepes/KOH, pH 7.5, 0.6 M sorbitol) containing 10 mM $Mg(OAc)_2$ and 40 mM KOAc were mixed with 10 μCi [^{35}S]cysteine (1075 Ci/mmol). Following incubation at 30 °C for different time periods (5–30 min), reaction mixtures were diluted with HS buffer containing 0.15 M NaCl and left on ice for 10 min. Samples were centrifuged at 15,000 × *g* for 10 min at 4 °C, and the pellets were analyzed by non-reducing SDS-PAGE followed by autoradiography. In some cases, mitochondria were recovered after incubation with [^{35}S]cysteine as described above and solubilized with the immunoprecipitation (IP) buffer (50 mM Tris/HCl, pH 7.5, 1% Triton X-100, 0.15 M NaCl, 1 mM PMSF). The mitochondrial lysate was centrifuged at 15,000 × *g* for 15 min at 4 °C to remove insoluble material, if any, and the supernatant was added to Protein A-Sepharose with prebound anti-Nfs1p antibodies. After incubation at 4 °C for 2 h, the beads were washed with the IP buffer, and the bound proteins were eluted with SDS loading buffer containing no DTT. Samples were analyzed by non-reducing SDS-PAGE followed by autoradiography. For some other experiments, mitochondria were pretreated with N-ethylmaleimide (NEM), DTT or *o*-phenanthroline prior to incubation with [^{35}S]cysteine. These differences are indicated in the corresponding figure legends.

To determine the cysteine desulfurase activity of the purified Nfs1p•Isd11p complex, the reaction mixture contained the protein complex (5–50 ng) in HS buffer containing 0.15 mM PLP, 0.15 M NaCl, and 10 μCi [^{35}S]cysteine in a final volume of 50 μl. Following incubation at 30 °C for 15 min, the reaction was stopped and proteins were precipitated with the addition of equal volume of ice-cold 20% trichloroacetic acid (TCA) followed by centrifugation at 15,000 × *g* for 30 min at 4 °C. The pellet samples were analyzed by non-reducing SDS-PAGE followed by autoradiography. The cysteine desulfurase activity of purified *A. vinelandii* NifS–His_6 was determined exactly the same way. In some cases, the purified Nfs1p•Isd11p complex or *A. vinelandii* NifS–His_6 was treated with NEM and/or DTT prior to incubation with [^{35}S]cysteine as indicated in the corresponding figure legends.

2.4. Insertion of radiolabeled clusters into aconitase

Isolated mitochondria contain a pool of apoaconitase that can be used as a substrate for Fe–S cluster loading. Assays were performed essentially as described (Amutha et al., 2008, 2009; Pain et al., 2010; Yoon et al.,

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