A NOVEL ROLE FOR HUMAN NFS1 IN THE CYTOPLASM: Nfs1 acts as a sulfur donor for MOCS3, a protein involved in molybdenum cofactor biosynthesis†

Zvonimir Marelja1, Walter Stöcklein1, Manfred Nimtz2, and Silke Leimkühler1*
1University of Potsdam, Institute of Biochemistry and Biology, D-14476 Potsdam, Germany; 2Helmholtz Center for Infection Research, 38124 Braunschweig, Germany.

Running title: Human Nfs1 acts as a sulfur donor for MOCS3 in Moco biosynthesis
*To whom correspondence should be addressed: Tel.: +49-331-977-5603; Fax: +49-331-977-5419; E-mail: sleim@uni-potsdam.de
†This work was supported by Deutsche Forschungsgemeinschaft Grant LE1171/5-3 and the Fonds der Chemischen Industrie.

The human MOCS3 gene encodes a protein involved in activation and sulfuration of the C-terminus of MOCS2A, the smaller subunit of the molybdopterin (MPT) synthase. MPT synthase catalyzes the formation of the dithiolene group of MPT that is required for the coordination of the molybdenum atom in the last step of molybdenum cofactor (Moco) biosynthesis. The two domain protein MOCS3 catalyzes both the adenylation and the subsequent generation of a thiocarboxylate group at the C-terminus of MOCS2A by its C-terminal rhodanese-like domain (RLD). The low activity of MOCS3-RLD with thiosulfate as sulfur donor and detailed mutagenesis studies showed that thiosulfate is most likely not the physiological sulfur source for Moco biosynthesis in eukaryotes. It was suggested that a L-cysteine desulfurase might be involved in the sulfuration of MOCS3 in vivo. In this report, we investigated the involvement of the human L-cysteine desulfurase Nfs1 in sulfur transfer to MOCS3-RLD. A variant of Nfs1 was purified in conjunction with Isd11 in a heterologous expression system in E. coli, and the kinetic parameters of the purified protein were determined. By studying direct protein-protein interactions, we were able to show that Nfs1 interacted specifically with MOCS3-RLD and that sulfur is transferred from L-cysteine to MOCS3-RLD via an Nfs1-bound persulfide intermediate. Since MOCS3 was shown to be located in the cytosol, our results suggest that cytosolic Nfs1 has an important role in sulfur transfer for the biosynthesis of Moco.

Among the metabolic pathways requiring sulfur transfer are those leading to the formation of iron sulfur (FeS) clusters, biotin, thiamine, lipoic acid, molybdopterin (MPT), and sulfur-containing bases in tRNA (1). MPT, the basic component of the molybdenum cofactor (Moco), is a tricyclic pterin derivative that bears the cis-dithiolene group essential for molybdenum ligation (2). Moco is essential for the activity of sulfite oxidase, xanthine dehydrogenase, and aldehyde oxidase in humans (3). The biosynthetic pathway of Moco can be divided into three steps: i) conversion of GTP to precursor Z; ii) transformation of precursor Z to MPT; and iii) insertion of molybdenum into MPT to form Moco (4).

Recent studies have identified the human genes involved in the biosynthesis of Moco (5). Human MPT synthase, like the E. coli (MoaD-MoaE)2 counterpart, is a heterotetramer which is composed of two so-called MOCS2A (~9,700 Da) and MOCS2B (~20,800 Da) subunits (6). The sulfur used to generate the dithiolene moiety of MPT is carried on the MOCS2A subunit in the form of a C-terminal thiocarboxylate, that must be regenerated during each catalytic cycle. The reaction mechanism of resulfuration of E. coli MPT synthase has been described in detail (7-10). Similar to ubiquitin activating enzymes (E1), the MOCS3 protein activates the C-terminus of MOCS2A to form an acyl adenylate (11). Subsequently, the MOCS2A acyl adenylate is converted to a thiocarboxylate by action of the C-terminal rhodanese-like domain (RLD) of MOCS3 (8). This RLD is present in all eukaryotic homologues, including the Saccharomyces cerevisiae Uba4 protein, and several bacterial homologues, but not in the E. coli MoeB protein (12). MOCS3 was shown to catalyze both the
adenylation and the subsequent generation of a thiocarboxylate group at the C-terminus of MOCS2A during Moco biosynthesis (13). All three proteins were shown to be localized in the cytosol in humans (14). In addition, a recent report showed that MOCS3 also catalyzes the adenylation and sulfuration of hUrm1, a protein suggested to be involved in protein-conjugation in humans (11). Rhodaneses (thiosulfate:cyanide sulfurtransferases, E.C. 2.8.1.1) are widespread enzymes that in vitro catalyze the transfer of a sulfane sulfur atom from thiosulfate to cyanide (15). They are not only found in combination with other proteins but also as single domain proteins or as tandem repeats serving as versatile sulfur carriers (15,16). A cysteine is the first residue of a six amino acid active-site loop defining the ridge of the catalytic pocket that is expected to play a key role in substrate recognition and catalytic activity (15). Detailed studies of the MOCS3-RLD sulfurtransferase activity showed that MOCS3 acts in vitro as a thiosulfate:cyanide sulfurtransferase, however, the activity was determined to be more than 1000-times lower compared to the activity of bovine rhodanese (14). A detailed mutagenesis study of the six amino acids active-site loop of MOCS3-RLD suggested that thiosulfate is most likely not the physiological sulfur source for MOCS3 in humans. It has been proposed that an L-cysteine desulfurase might act as direct sulfur donor for cytosolic MOCS3 in humans (14). L-cysteine desulfurases are pyridoxal phosphate (PLP) containing enzymes that catalyze the formation of L-alanine and a protein-bound persulfide group by using L-cysteine as substrate (17,18).

A single L-cysteine desulfurase homologue, named Nfs1, was identified in humans (19,20). However, it was shown that two distinct Nfs1 isoforms are produced through alternative utilization of in-frame AUGs (19,21,22). The major form is generated by initiation of the first AUG of the Nfs1 transcript and contains a mitochondrial targeting signal at the N-terminus that undergoes cleavage to yield a mature mitochondrial protein of 47 kDa in size (19). In mitochondria, Nfs1 is involved in FeS cluster biosynthesis (23). A less abundant isoform generated by initiation of translation at the second in-frame AUG lacks the first 60 residues of the mitochondrial precursor form, and this 44 kDa protein resides both in the cytosol and in the nucleus (19).

Recently, the Isd11 protein was identified to be essential for FeS cluster biosynthesis in mitochondria and it was shown that Isd11 forms a complex with Nfs1 (24,25). Isd11 is suggested to function as an adapter and stabilizer of Nfs1 (26). Homologues of Isd11 have been identified in plant, fungi and animal genomes, which contain mitochondria, but no prokaryotic homologue has been identified (27).

To analyze whether Nfs1 acts as sulfur donor for the biosynthesis of the molybdenum cofactor, we purified a N-terminal truncated version of Nfs1 after heterologous expression in E. coli in the presence and absence of Isd11. The L-cysteine desulfurase activity of the purified Nfs1Δ1-55/Isd11 complex was characterized. Nfs1Δ1-55 was shown to interact with both Isd11 and MOCS3-RLD, and in addition, the interaction with MOCS3-RLD was stronger when Nfs1Δ1-55 was not in a complex with Isd11. By ESI-MS analyses it was verified that the sulfur from Nfs1 is further transferred to form a persulfide on MOCS3-RLD. Our studies suggest that cytosolic Nfs1 has an additional role in the cytosol and sulfurates MOCS3 for the biosynthesis of the molybdenum cofactor.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, Media and Growth Conditions -** E. coli BL21(DE3) cells or BL21(DE3)star cells (Novagen) were used for heterologous expression of the human Nfs1Δ1-55 and Isd11 proteins. The vectors pET15b and pACYC-Duet-1 were obtained from Novagen. E. coli expression cultures were grown in LB medium under aerobic conditions at 16°C for 16 h. Ampicillin (150 µg/mL), chloramphenicol (50 µg/mL), and isopropyl-β-D-thiogalactoside (IPTG) (100 µM) were used when required.

**Cloning, Expression and Purification of human Nfs1 and Isd11** – For expression of Nfs1, primers were designed that resulted in a deletion of the first 55 amino acids of Nfs1 and that allowed cloning into the XhoI-BamHI sites of the expression vector pET15b. The resulting plasmid was designated pZM2, and expresses Nfs1Δ1-55 as a N-terminal fusion protein with a His6-tag. For coexpression with Isd11, the Isd11 cDNA fragment was cloned from a human cDNA library. Primers were designed that allowed cloning of Isd11 into the Neol-HindIII sites of the expression vector pACYC-Duet-1. The resulting plasmid was
designated pZM4. For separate purification of Isd11, primers were designed that allowed cloning of Isd11 into the Xhol-BamHI sites of the expression vector pET15b. The resulting plasmid was designated pZM6, and expresses Isd11 as a N-terminal fusion protein with a His6-tag.

For purification of Nfs1Δ1-55, plasmid pZM2 was cotransformed with a plasmid containing the E. coli chaperonin GroEL (6) in E. coli BL21(DE3)star cells. For heterologous expression of Nfs1Δ1-55/Isd11 in E. coli, pZM2 and pZM4 were cotransformed into BL21(DE3)star cells. Cells were grown at 30°C in 1 L cultures of LB containing 150 μg/mL ampicillin and 50 μg/mL chloramphenicol. The expression was induced at OD600nm=0.6 with 100 μM IPTG. Growth was continued for 16 h at 16°C, and cells were harvested by centrifugation at 8000 × g. After cell lysis, the soluble fraction was transferred onto a column with Ni-nitritoltriacetic (Ni-NTA, Qiagen). Nfs1Δ1-55 or Nfs1Δ1-55/Isd11 were eluted with 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole buffer (pH 8.0), containing 50 μM PLP and 10% (v/v) glycerol. Final purification of Nfs1Δ1-55 or Nfs1Δ1-55/Isd11 was achieved by chromatography on a Superose 12 size exclusion column (GE Healthcare) equilibrated in 50 mM Tris, 200 mM NaCl, 10 μM PLP (pH 8.0).

For expression of Isd11, pZM6 was transformed into E. coli BL21(DE3) cells. Cells were grown at 30°C in 1 L cultures of LB, containing 150 μg/mL ampicillin. The expression was induced at OD600nm=0.6 with 100 μM IPTG. Growth was continued for 16 h at 16°C, and cells were harvested by centrifugation at 8000 × g. After cell lysis, the soluble fraction was transferred onto a column with Ni-NTA. Isd11 was eluted with 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole buffer (pH 8.0). Final purification of Isd11 was achieved by chromatography on a Superose 12 size exclusion column equilibrated in 50 mM Tris, 200 mM NaCl (pH 9.0).

Size Exclusion Chromatography - Purified Nfs1Δ1-55 or Nfs1Δ1-55/Isd11 were injected in a volume of 500 μL onto a Superdex 200 column (GE Healthcare) equilibrated in 50 mM Tris, 200 mM NaCl, 1 mM EDTA (pH 8.0).

Enzyme assays, molar extinction coefficient and absorption spectra of Nfs1Δ1-55 – L-cysteine desulfurase activity of Nfs1Δ1-55/Isd11 was quantified by the methylene blue method (28) using the parameters from Urbina et al. (29). Assay mixtures in a total volume of 0.8 mL contained 50 mM Tris, 200 mM NaCl, 10 μM PLP, 1 mM DTT (pH 8.0), and 2 μM of Nfs1Δ1-55/Isd11. The reactions were initiated by the addition of L-cysteine (0.1 -1.0 mM) and incubated for 10 min at 37°C before the reactions were stopped by the addition of 100 μL 20 mM N,N-dimethyl-p-phenylenediamine in 7.2 M HCl and 100 μl 30 mM FeCl₃ in 1.2 M HCl. After an incubation time of 15 min, samples were centrifuged for 5 min at 12,000 × g. The supernatant was transferred to cuvettes and methylene blue was determined at 670 nm. The standard curve was recorded with sodium sulfide. Nfs1Δ1-55 concentrations were determined from the absorbance at 420 nm using the extinction coefficient of 10.9 mM⁻¹ cm⁻¹ for the native enzyme. The extinction coefficient was determined on the basis of the PLP content after alkaline denaturation described after the method of Peterson and Sober (30).

The reduction spectrum of 9 μM Nfs1Δ1-55/Isd11 or 2 μM Nfs1Δ1-55 were recorded in the presence of 10 mM L-cysteine in 50 mM Tris, 1 mM EDTA, 200 mM NaCl, pH 8.0 at room temperature using a UV-2401PC Shimadzu spectrometer.

Surface Plasmon Resonance (SPR) Measurements – All binding experiments were performed with the SPR-based instrument Biacore™ 2000 on CM5 sensor chips at a temperature of 25°C and a flow rate of 10 μL/min, using the control software 2.1 and evaluation software 3.0 (Biacore AB, Uppsala, Sweden) as described previously (31). The autosampler-racks containing the sample vials were cooled to 4°C. Immobilization of proteins yielded about 700 – 3,300 resonance units (RU) per flow cell (BSA: 1,530 RU; Isd11: 700 RU; MOCS3-RLD: 3,360 RU; Uba4: 1,870 RU).

As running buffer 20 mM phosphate, 150 mM NaCl, 0.005% Tween 20 (pH 7.4) was used. Nfs1Δ1-55 and Nfs1Δ1-55/Isd11 with concentrations of 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 μM were injected for 4.5 min at a flow rate of 30 μL/min followed by 15 min dissociation using thekinject command and regeneration of the sensor surface with 50 mM HCl for 1 min. Binding curves were corrected by subtraction of buffer injection curves for all four flow cells and curves of Nfs1Δ1-55 and Nfs1Δ1-55/Isd11 for the control flow cell.

Detection of sulfur transfer from Nfs1Δ1-55/Isd11...
**RESULTS**

**Purification of Nfs1Δ1-55, Isd11 and copurification of the Nfs1Δ1-55/Isd11 complex.**

For purification of Nfs1, the Nfs1 gene coding for amino acids 56-458 was cloned into the *E. coli* pET15b expression vector, resulting in a N-terminal His<sub>6</sub>-tagged recombinant protein. To increase protein stability, *E. coli* groEL genes were coexpressed with Nfs1Δ1-55. The soluble fraction of Nfs1Δ1-55 was purified by Ni-NTA chromatography and size exclusion chromatography on a Superose 12 column, and after elution one major band was visible on SDS-polyacrylamide gels with a size, corresponding closely to the calculated molecular mass of 47.3 kDa for His<sub>6</sub>-tagged Nfs1Δ1-55 (Fig. 1A). Densitometric analyses showed that the protein was 65% pure. The additional band visible on the SDS polyacrylamide gel was determined by MALDI-peptide mapping and confirmed by MS/MS analyses to be the *E. coli* SlyD protein, a histidine-rich FKBP-type peptidyl-prolyl cis-trans isomerase, a protein that is often copurified with recombinant proteins by Ni-NTA chromatography. The purified Nfs1Δ1-55 protein was stable for about 2 days at 4°C. In contrast, the full-length Nfs1 protein could not be purified since the majority of the protein was unstable or existed in inclusion bodies after expression in *E. coli*. Coexpression of GroEL resulted in a higher yield of soluble Nfs1Δ1-55, likely by preventing the formation of inclusion bodies during expression. In contrast, the stability of the purified protein was not influenced by the presence of GroEL during expression, as shown by thermal denaturation studies using circular dichroism spectroscopy (see Fig. S1, which is published as supplemental data on the JBC web site).

For purification of Isd11, the gene *Isd11* was cloned into the *E. coli* pET15b expression vector, resulting in the N-terminal His<sub>6</sub>-tagged recombinant protein. Ni-NTA affinity chromatography and size exclusion chromatography on a Superose 12 column was carried out to obtain a purified Isd11 in a soluble form. The purified Isd11 displayed a single band on Coomassie Brilliant Blue-stained SDS-polyacrylamide gels with a monomeric mass corresponding to the calculated mass of the His<sub>6</sub>-tagged Isd11 of 13.4 kDa (Fig. 1C).

To increase the stability of Nfs1Δ1-55, the Isd11 gene was cloned into a vector containing the P15A origin and coexpressed with Nfs1Δ1-55 in *E. coli* cells. The Nfs1Δ1-55/Isd11 complex was purified
by Ni-NTA and size exclusion chromatography on a Superose 12 column, and displayed two bands on Coomassie Brilliant Blue-stained SDS-polyacrylamide gels (Fig. 1B), corresponding to Nfs1Δ1-55 and Isd11, respectively. The presence of Isd11 increased the stability of Nfs1 significantly, the protein was stable for several days at 4°C and did not lose activity after storage at -20°C. As shown by thermal denaturation studies by circular dicroism spectroscopy, the protein also displayed a better stability at higher temperatures in comparison to Nfs1Δ1-55 expressed in the absence of Isd11 (see Fig. S1, which is published as supplemental data on the JBC web site).

To identify the oligomerization state of Nfs1Δ1-55 and the Nfs1Δ1-55/Isd11 complex, the purified proteins were subjected to size exclusion chromatography. The observed elution position of Nfs1Δ1-55 from a Superdex 200 column corresponded to a mass of 90 kDa (Fig. 2A), showing that the protein existed as a dimer in solution. In comparison to proteins with a similar size, the observed elution position of the Nfs1Δ1-55/Isd11 complex revealed a mass of approximately 250 kDa on the analytical size exclusion column (Fig. 2B), at least corresponding to a (Nfs1Δ1-55/Isd11)₂ octamer.

Functional complementation of the E. coli CL100(iscS) strain and analysis of the kinetic parameters of the Nfs1Δ1-55/Isd11 complex.

Nfs1 shares an amino acid sequence identity with E. coli IscS of 60%. Thus, we analyzed whether Nfs1 was able to functionally complement the E. coli CL100(iscS) strain (33). The E. coli CL100(iscS) strain was either transformed with the plasmid containing Nfs1Δ1-55 or cotransformed with the Nfs1Δ1-55 and Isd11 containing plasmids and cells were grown at 30°C in the presence of IPTG over a period of 8 h. The corresponding growth curves showed that Nfs1Δ1-55 was able to complement the role of IscS in the E. coli CL100(iscS) strain both in the presence or absence of Isd11, showing that the Nfs1Δ1-55 was expressed in a functional form (data not shown).

The purified Nfs1Δ1-55 protein exhibited the characteristic yellow color observed for other L-cysteine desulfurases containing PLP as prosthetic group. UV-VIS absorption spectra of the purified Nfs1Δ1-55 and the Nfs1Δ1-55/Isd11 complex were similar and exhibited the absorption maximum at 420 nm (Fig. 3A and B), characteristic for L-cysteine desulfurases (17). The addition of 10 mM L-cysteine resulted in a decrease of absorbance at 420 nm and an increase of absorbance at 320 nm of both purified Nfs1Δ1-55 and Nfs1Δ1-55/Isd11 (Fig. 3), showing that both proteins were reduced by L-cysteine. However, Nfs1Δ1-55 was only able to perform one turnover and precipitated rapidly after reduction (Fig. 3A, data not shown).

To determine the kinetic parameters of Nfs1Δ1-55, steady state kinetics were performed by varying the concentration of L-cysteine using the purified Nfs1Δ1-55 protein and the Nfs1Δ1-55/Isd11 complex. The pH optimum of Nfs1Δ1-55/Isd11 was determined to be at 8.0 and the temperature optimum was at 46°C (data not shown). Enzyme assays were performed at 37°C by varying the concentrations of L-cysteine and enzyme activity was determined using the methylene blue method, detecting the release of H₂S in the assay (28). Enzyme activity was only detectable for the Nfs1Δ1-55/Isd11 complex. For Nfs1Δ1-55, no enzyme activity could be detected even at lower temperatures (data not shown). This is consistent with the observation, that Nfs1Δ1-55 is rather unstable after reduction and precipitated under the assay conditions. Thus, the apparent kinetic constants were only determined for Nfs1Δ1-55/Isd11 and showed a Kₘ of 434.75 μM and a kₗ of 2.5 min⁻¹.

Analysis of protein-protein interactions by Surface Plasmon Resonance Measurements.

After purification of Nfs1Δ1-55 it was of interest to determine whether the protein is able to interact with MOCS3-RLD in vitro. SPR measurements were employed for real time detection of specific interactions using the purified proteins. All proteins were immobilized on the CM5 chip via amine coupling.

To analyze the functionality of the purified Nfs1Δ1-55 protein and of the Nfs1Δ1-55/Isd11 complex, their ability to interact with immobilized Isd11 was analyzed. The results obtained by SPR measurements for the protein pairs are listed in Table 1. While Nfs1Δ1-55 was able to bind to immobilized Isd11 with a Kₐ of 100 nM, the Nfs1Δ1-55/Isd11 complex did not bind to immobilized Isd11, showing that all binding sites on purified Nfs1Δ1-55 were occupied with Isd11. This also showed that the purified Nfs1Δ1-55
protein was functional in the binding experiments and could be used for further analyses.

Since a functional full-length MOCS3 protein is not available in a purified form so far, the interaction of Nfs1Δ1-55 and MOCS3 was analyzed with the immobilized MOCS3-RLD protein. In order to differentiate whether Nfs1Δ1-55 interacts solely with the C-terminal rhodanese-like domain of MOCS3 for sulfur transfer, or whether the N-terminus is important for the interaction, the MOCS3-homologue Uba4 from *S. cerevisiae* was used for comparison (11). As shown in Table 1, Nfs1Δ1-55 interacted with immobilized MOCS3-RLD or Uba4 with comparable K_D values of 86 nM and 84 nM, respectively. In contrast, the interaction of the Nfs1Δ1-55/Isd11 complex with both proteins was weaker, showing K_D values of 480 nM with MOCS3-RLD and 610 nM with Uba4. These results showed that Nfs1Δ1-55 interacted with both MOCS3-RLD and Uba4 and that the interaction most likely occurred via the C-terminal RLD-domain of both proteins. Also, the Nfs1Δ1-55/Isd11 complex interacted less tightly with both MOCS3-RLD and Uba4, since most likely Isd11 interacts with the binding sites for MOCS3-RLD and Uba4.

**Analysis of sulfur transfer from Nfs1Δ1-55/Isd11 to MOCS3-RLD with L-cysteine as sulfur source.**

To test whether the protein-bound persulfide group can be transferred from Nfs1Δ1-55 further onto MOCS3-RLD, L-cysteine, Nfs1Δ1-55/Isd11 and MOCS3-RLD were incubated. The sulfur on MOCS3-RLD was determined by the production of thiocyanate using cyanide as substrate, since it was shown before that cyanide has a higher affinity to rhodanases than to L-cysteine desulfurases (34). The released thiocyanate can be quantified by the colorimetric method described by Sörbo (32). Fig. 4 shows the time course of the incubation mixtures in comparison to the control reactions. While the incubation mixture containing MOCS3-RLD, Nfs1Δ1-55/Isd11, and L-cysteine showed the highest rate of thiocyanate formation, the mixture containing solely L-cysteine and Nfs1Δ1-55/Isd11 also produced thiocyanate, but with a slower rate. The control reaction containing L-cysteine and MOCS3-RLD confirmed that MOCS3-RLD does not use L-cysteine as a sulfur source (14). Also the rate of thiocyanate formation by MOCS3-RLD with thiosulfate as sulfur donor was significantly slower in comparison to the reaction containing L-cysteine and Nfs1Δ1-55, which is in contradiction to the hypothesis that thiosulfate is the physiological sulfur donor for MOCS3-RLD.

**Detection of the persulfide group on MOCS3-RLD by ESI-MS.**

To verify the results of the sulfur transfer from Nfs1Δ1-55/Isd11 to MOCS3-RLD using L-cysteine as sulfur source, the persulfide group on MOCS3-RLD was directly identified by ESI-MS. After incubation of MOCS3-RLD with Nfs1Δ1-55/Isd11 and L-cysteine, a mass increase of 32 Da was detected on MOCS3-RLD, corresponding to the addition of a sulfur atom (Fig. 5A). In comparison, incubation of MOCS3-RLD with L-cysteine, did not result in a mass increase of MOCS3-RLD (Fig. 5B), while the incubation of MOCS3-RLD with thiosulfate showed the expected mass increase of 32 Da (Fig. 5C). To confirm that Nfs1Δ1-55/Isd11 transfers the sulfur to the conserved cysteine residue Cys412 of MOCS3-RLD, the variant MOCS3-RLD-C412A was incubated with Nfs1Δ1-55/Isd11 and L-cysteine. In the ESI-MS spectrum of MOCS3-RLD-C412A no mass increase was observed (data not shown), showing that Nfs1Δ1-55/Isd11 specifically transfers the sulfur to the cysteine in the active-site loop of MOCS3-RLD.

**DISCUSSION.**

L-cysteine desulfurases are versatile proteins. In bacteria, the involvement of L-cysteine desulfurases were identified in the biosynthesis of FeS clusters, thiamine, thionucleosides in tRNA, biotin, and lipoic acid (18). Bacterial L-cysteine desulfurases are encoded by operons specifying the nitrogen fixation (NIF) machinery, the iron-sulfur cluster (ISC) assembly machinery, the sulfur mobilization (SUF) machinery and the CSD system (35-39). For bacterial Moco biosynthesis of the *E. coli* system, it has been shown that any of the three L-cysteine desulfurases IscS, CsdA, and SufS can act as a sulfur donor for the formation of the thioocarboxylate group on MoaD, the small subunit of MPT synthase (8). This sulfur is further used for the generation of the dithiolenone group of MPT. Thus, L-cysteine desulfurases might also be involved in the sulfur transfer for Moco biosynthesis *in vivo*, however, the specific L-
cysteine desulfurase has not been identified in E. coli so far (8).

In eukaryotes, the L-cysteine desulfurase Nfs1 was initially detected in mitochondria to be essential for FeS cluster biosynthesis, but there is also a cytosolic/nuclear form of Nfs1 in yeast and humans, which is required for cell viability and for post-translational modification of tRNAs in the nucleus (19,20,40-43). In humans, it is believed that FeS cluster biosynthesis is compartmentalized, since copies of Nfs1 and IscU are also found in the cytosol, which are derived by usage of alternative start codons in the transcript of these genes (19,23,44). Recently, a protein essential for the activity of Nfs1 has been identified in mitochondria, which was named Isd11 (24,25). Nfs1 was shown to form a tight complex with Isd11, thus stabilizing Nfs1 and preventing its aggregation. It was also reported that Isd11 binds to frataxin, and it was suggested that especially the complex formation between frataxin and Nfs1/IscU is mediated through Isd11 (26). A homologue of Isd11 was not identified in prokaryotes (27).

Here, we report the purification and characterization of a truncated version of Nfs1, Nfs1Δ1-55, which was expressed in a heterologous E. coli system in the presence or absence of Isd11. In the presence of Isd11, a stable Nfs1Δ1-55/Isd11 complex was formed. It was shown that Isd11 stabilized Nfs1Δ1-55 significantly in vitro. This is the first report of the purification and characterization of the Nfs1Δ1-55/Isd11 complex in addition to separately purified Isd11 and Nfs1Δ1-55. So far, it was not possible to purify mitochondrial and cytosolic Nfs1 after heterologous expression in bacteria, since the protein was sequestered in inclusion bodies (21). The deletion of the first 55 N-terminal amino acids of the mitochondrial targeting sequence led to the purification of an active protein, which was stable for 2 days at 4°C, but precipitated during turnover under assay conditions. The presence of Isd11 increased the stability of the Nfs1Δ1-55 significantly and enabled detailed studies of the protein. The determined k_cat in this report with L-cysteine for Nfs1Δ1-55/Isd11 is about 8.5 times higher than the value reported by Li et al. (21), using the cytosolic version of Nfs1 after expression in a heterologous Pichia pastoris expression system. In addition, the k_cat was about 4.3 times higher in comparison to the value reported for the purified S. cerevisiae Nfs1p protein (45), and about 3.4 times lower in comparison to the value of the E. coli IscS protein (29). This shows that the Nfs1Δ1-55/Isd11 complex was purified in a highly active form. Size exclusion chromatography showed that Nfs1 forms a dimer in solution, which is consistent with the oligomerization state of other L-cysteine desulfurases. However, in complex with Isd11, the size of the protein multimer increased to a size of about 250 kDa, showing an oligomerization state of the protein complex corresponding to at least a (Nfs1Δ1-55/Isd11)_2 octamer. A higher oligomerization state of Isd11 is also possible in the protein complex. Our result is consistent with previous reports, where a size of about 230 kDa was identified for the purified Nfs1/Isd11 complex from yeast mitochondria (24,25).

In analogy to the bacterial system, we wanted to analyze whether Nfs1 can act as a sulfur donor for MOCS3, involved in human Moco biosynthesis. Since the proteins for the second step of Moco biosynthesis, MOCS2A, MOCS2B and MOCS3 are located in the cytosol (14), this would imply a novel function for cytosolic Nfs1 in humans. Human MOCS3 contains a C-terminal rhodanese-like domain which was shown to act as direct sulfur donor for the formation of the thio-carboxylate group on human MOCS2A (13,14). However, the low activity of MOCS3 with thiosulfate and detailed mutagenesis studies of the rhodanese active-site loop suggested that thiosulfate is not the physiological sulfur source of MOCS3, and that proteins like L-cysteine desulfurases might perform this function in humans (12). SPR measurements showed that Nfs1Δ1-55 interacted with MOCS3-RLD both in the presence and absence of Isd11, however, the interaction with MOCS3-RLD was significantly stronger when Isd11 was absent. Thus, Isd11 seems to interfere with the interaction site of MOCS3 on Nfs1. Since Isd11 was so far only determined in mitochondria, the interaction between Nfs1 and MOCS3 in the cytosol might occur without the involvement of Isd11, thus in vivo there is no competition between Isd11 and MOCS3 for binding to Nfs1. By comparison of the binding of MOCS3-RLD and Uba4 to Nfs1Δ1-55, it became clear that the interaction site on MOCS3 must be located at its C-terminal rhodanese-like domain. By ESI-MS analyses it was directly confirmed that Nfs1 transferred the sulfur to...
MOCS3-RLD using L-cysteine as sulfur donor. The rate of MOCS3-RLD sulfuration by Nfs1 was about two times higher in comparison to thiosulfate as sulfur source. Unfortunately, due to the low stability of the Nfs1Δ1-55 protein, the sulfuration experiments could only be performed using the Nfs1Δ1-55/Isd11 complex. However, it is expected that the rate of sulfur transfer of Nfs1Δ1-55 to MOCS3-RLD in the absence of Isd11 is even higher, since the binding constant between the two proteins was 5.6 times higher in the absence of Isd11.

In total, we suggest that human Nfs1 acts as direct sulfur donor for the biosynthesis of Moco in the cytosol. The sulfur transfer might occur as suggested in the model in Fig. 6: MOCS3 activates MOCS2A in the presence of ATP [1] by formation of an acyl-adenylate bond [2]. MOCS2A is further transferred to a persulfide group on Cys412 of MOCS3-RLD, forming a disulfide bond [3]. Release of the MOCS2A thiocarboxylate requires a second thiol group, which is proposed to be MOCS3-Cys239 [3], but other reducing factors are also possible. MOCS2A-thiocarboxylate is released and an intramolecular disulfide group is formed on MOCS3 [4], which is further reduced e.g. by the thioredoxin system in the cell ([5], hypothetical) (14). The persulfide on MOCS3-RLD is formed by interaction with Nfs1, and the sulfur atom of Nfs1 is transferred by a nucleophilic attack of the MOCS3-Cys412 cysteinate on the persulfide sulfur of Nfs1-Cys381 [6]. After the reaction, Nfs1 in turn catalyzes the desulfuration of L-cysteine to L-alanine for the formation of a novel intramolecular persulfide group on Cys381 [7]. Finally, persulfide-containing MOCS3 is formed [8], which can react again with MOCS2A and ATP [1].

In humans, active Moco is essential for the activity of sulfite oxidase, xanthine oxidoreductases and aldehyde oxidase (46). Moco deficiency leads to the pleiotropic loss of all these molybdoenzymes and usually progresses to death at an early age (47). The clinical symptoms displayed by the affected neonates include feeding difficulties and neurological abnormalities such as attenuated brain growth, untreatable seizures, dislocated ocular lenses and in most cases, death in early childhood (48). The clinical symptoms result from an accumulation of sulfite in the body, due to the loss of sulfite oxidase activity, which catalyzes the detoxification of sulfite to sulfate (3). In contrast, in S. cerevisiae, where Nfs1p has a major role in mitochondria for FeS cluster biosynthesis (45), the cytosolic form of Nfs1p might be not as important as in humans, since S. cerevisiae is one of the few organisms that do not contain molybdoenzymes or a machinery for Moco biosynthesis (49).

REFERENCES:
1. Marquet, A. (2001) Curr Opin Chem Biol 5, 541-549
2. Rajagopalan, K. V., Johnson, J. L., and Hainline, B. E. (1982) Fed. Proc. 41, 2608-2612
3. Johnson, J. L., and Duran, M. (2001) Molybdenum cofactor deficiency and isolated sulfite oxidase deficiency. In: Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Childs, B., and Vogelstein, B. (eds). The Metabolic and Molecular Bases of Inherited Disease, 8th edition, McGraw-Hill, New York
4. Rajagopalan, K. V. (1996) Biosynthesis of the molybdenum cofactor. In: Neidhardt, F. C. (ed). Escherichia coli and Salmonella. Cellular and Molecular Biology, ASM Press, Washington, DC
5. Reiss, J. (2000) Hum Genet 106, 157-163
6. Leimkühler, S., Freuer, A., Santamaria Araujo, J. A., Rajagopalan, K. V., and Mendel, R. R. (2003) J Biol Chem 278, 26127-26134
7. Lake, M. W., Wuebbens, M. M., Rajagopalan, K. V., and Schindelin, H. (2001) Nature 414, 325-329
8. Leimkühler, S., and Rajagopalan, K. V. (2001) J. Biol. Chem. 276, 22024-22031
9. Leimkühler, S., Wuebbens, M. M., and Rajagopalan, K. V. (2001) J. Biol. Chem. 276, 34695-34701
10. Wuebbens, M. M., and Rajagopalan, K. V. (2003) J Biol Chem 278, 14523-14532
11. Schmitz, J., Mullich Cowdhury, M., Hänzelmann, P., Lee, E.-Y., Schindelin, H., and Leimkühler, S. (2008) *Biochemistry* **47**, 4679-89
12. Krepinsky, K., and Leimkühler, S. (2007) *FEBS J.* **274**, 2778-2787
13. Matthies, A., Nimtz, M., and Leimkühler, S. (2005) *Biochemistry* **44**, 7912-7920
14. Matthies, A., Rajagopalan, K. V., Mendel, R. R., and Leimkühler, S. (2004) *Proc Natl Acad Sci U S A* **101**, 5946-5951
15. Bordo, D., and Bork, P. (2002) *Embo reports* **3**, 741-746
16. Mueller, E. G. (2006) *Nat Chem Biol* **2**(4), 185-194
17. Zheng, L., White, R. H., Cash, V. L., Jack, R. F., and Dean, D. R. (1993) *Proc Natl Acad Sci U S A* **90**(7), 2754-2758.
18. Mihara, H., and Esaki, N. (2002) *Appl Microbiol Biotechnol* **60**, 12-23
19. Land, T., and Rouault, T. A. (1998) *Mol cell* **2**, 807-815
20. Biederbick, A., Stehling, O., Rosser, R., Niggemeyer, B., Nakai, Y., Elsasser, H. P., and Lill, R. (2006) *Mol Cell Biol* **26**(15), 5675-5687
21. Li, K., Tong, W. H., Hughes, R. M., and Rouault, T. A. (2006) *J Biol Chem* **281**(18), 12344-12351
22. Tong, W. H., and Rouault, T. (2000) *Embo J* **19**(21), 5692-5700.
23. Tong, W. H., and Rouault, T. A. (2006) *Cell Metab* **3**(3), 199-210
24. Adam, A. C., Bornhovd, C., Prokisch, H., Neupert, W., and Hell, K. (2006) *Embo J* **25**(1), 174-183
25. Wiedemann, N., Urzica, E., Guiard, B., Müller, H., Lohaus, C., Meyer, H. E., Ryan, M. T., Meisinger, C., Mühlenhoff, U., Lill, R., and Pfänner, N. (2006) *Embo J* **25**(1), 184-195
26. Shan, Y., Napoli, E., and Cortopassi, G. (2007) *Hum Mol Genet* **16**(8), 929-941
27. Richards, T. A., and van der Giezen, M. (2006) *Mol Biol Evol* **23**(7), 1341-1344
28. Fogo, J. K., and Popowsky, M. (1949) *Anal Chem* **21**, 732-734
29. Urbina, H. D., Silberg, J. J., Hoff, K. G., and Vickery, L. E. (2001) *J Biol Chem* **276**(48), 44521-44526
30. Peterson, E. A., and Sober, H. A. (1954) *J Am Chem Soc* **76**, 169-175
31. Neumann, M., Schulte, M., Jünemann, N., Stöcklein, W., and Leimkühler, S. (2006) *J Biol Chem* **281**(23), 15701-15708
32. Sörbo, B. (1957) *Biochim Biophys Acta* **23**(2), 412-416
33. Lauhon, C. T., and Kambarpati, R. (2000) *J Biol Chem* **275**(26), 20096-20103.
34. Zheng, L., White, R. H., Cash, V. L., and Dean, D. R. (1994) *Biochemistry* **33**(15), 4714-4720.
35. Zheng, L., Cash, V. L., Flint, D. H., and Dean, D. R. (1998) *J Biol Chem* **273**(21), 13264-13272.
36. Takahashi, Y., and Tokumoto, U. (2002) *J Biol Chem* **277**(32), 28380-28383.
37. Outten, F. W., Djaman, O., and Storz, G. (2004) *Mol Microbiol* **52**(3), 861-872
38. Loiseau, L., Ollagnier-de Choudens, S., Lascoux, D., Forest, E., Fontecave, M., and Barras, F. (2005) *J Biol Chem* **280**(29), 26760-26769
39. Frazzon, J., and Dean, D. R. (2003) *Curr Opin Chem Biol* **7**(2), 166-173
40. Lill, R., and Mühlenhoff, U. (2008) *Annu Rev Biochem* in press
41. Lill, R., and Mühlenhoff, U. (2006) *Annu Rev Cell Dev Biol* **22**, 457-486
42. Nakai, Y., Nakai, M., Lill, R., Suzuki, T., and Hayashi, H. (2007) *Mol Cell Biol* **27**(8), 2841-2847
43. Lill, R., Dutkiewicz, R., Elsasser, H. P., Hausmann, A., Netz, D. J., Pierik, A. J., Stehling, O., Urzica, E., and Mühlenhoff, U. (2006) *Biochim Biophys Acta* **1763**(7), 652-667
44. Rouault, T. A., and Tong, W. H. (2005) *Nat Rev Mol Cell Biol* **6**(4), 345-351
Δ

(B), Spectra of 14.5

nate was quantified after the method of Sörbo

alyzed

aphy; (C) Isd11:

act after cell lysis,

Analysis of thiocyanate producti

Figure 6:

Figure 5:

Figure 4:

Figure 3:

Figure 2:

Figure 1:

FIGURE LEGENDS

Figure 1: Purification of Nfs1\^A1-55, Nfs1\^A1-55/Isd11 and Isd11 after heterologous expression in E. coli. 15% SDS-PAGE analysis of purification stages of (A) Nfs1\^A1-55: I, crude extract after cell lysis, and 2, 2 µg of Nfs1\^A1-55 after purification by Ni-NTA and Superose 12 chromatography; (B) Nfs1\^A1-55/Isd11: I, crude extract after cell lysis, 2, 2 µg of protein after Ni-NTA, and 3, 2 µg of Nfs1\^A1-55/Isd11 after Superose 12 chromatography; (C) Isd11: I, crude extract after cell lysis, and 2, 2 µg of Isd11 after Ni-NTA and Superose 12 chromatography.

Figure 2: Size exclusion chromatography of Nfs1\^A1-55 and Nfs1\^A1-55/Isd11. (A) 27 µM of Nfs1\^A1-55 and (B) 3 µM of Nfs1\^A1-55/Isd11 were analyzed by analytical size exclusion chromatography in 50 mM Tris, 1 mM EDTA, 200 mM NaCl, pH 8.0, using a Superdex 200 column. Inset, Plot of the standard proteins. Size exclusion chromatography markers (Biorad): gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (1.3 kDa).

Figure 3: Characterization of Nfs1\^A1-55/Isd11 by UV-VIS absorption spectroscopy. (A), Spectra of 14.5 µM of air-oxidized Nfs1\^A1-55 (solid line) and of the reduced enzyme with 20 mM L-cysteine (dashed line). (B), Spectra of 14.5 µM of air-oxidized Nfs1\^A1-55/Isd11 (solid line) and of the reduced enzyme with 20 mM L-cysteine (dashed line). Spectra were recorded in 50 mM Tris, 1 mM EDTA, 200 mM NaCl, 10 µM PLP, pH 8.0.

Figure 4: Analysis of thiocyanate production of MOCS3-RLD. Reaction mixtures contained: I, 2 µM MOCS3-RLD, 2 µM Nfs1\^A1-55/Isd11, and 10 mM L-cysteine (●); 2, 2 µM Nfs1\^A1-55/Isd11, and 10 mM L-cysteine (▲); 3, 2 µM MOCS3-RLD and 10 mM thiosulfate (♦); or 4, 2 µM MOCS3-RLD, and 10 mM L-cysteine (■). The mixtures were incubated in the presence of 30 mM KCN for the indicated time points, and produced thiocyanate was quantified after the method of Sörbo (32).

Figure 5: Deconvoluted ESI mass spectra of MOCS3-RLD. (A) MOCS3-RLD after incubation with Nfs1\^A1-55/Isd11 and L-cysteine; (B) MOCS3RLD after incubation with L-cysteine, and (C) MOCS3-RLD after incubation with thiosulfate. The mixtures were incubated in 50 mM Hepes, 200 mM NaCl, pH 8.0 and contained 20 µM Nfs1\^A1-55/Isd11, 50 µM MOCS3-RLD, 2 mM L-cysteine or 2 mM thiosulfate. Prior to incubation, MOCS3-RLD was treated with KCN. ESI-MS spectra were recorded in 3 mM NH\(^{4}\)OAc, pH 8.0. The mass of 19753.4 – 19755.5 Da corresponds to MOCS3-RLD and the mass of 19785.5 – 1987.9 Da with an increase of +32 Da to persulfurated MOCS3-RLD.

Figure 6: Proposed mechanism of the sulfur transfer involving MOCS3 and Nfs1 in humans. A detailed description of the mechanism is given in the text.

FOOTNOTES

The abbreviations used are: Moco, molybdenum cofactor; MPT, molybdopterin; Ni-NTA, nickel-nitrotriacetic acid; IPTG, isopropyl thio-β-D-galactoside; RLD, rhodanese-like domain; FeS, iron-sulfur.
Table I. **Analysis of protein-protein interactions between Nfs1Δ1-55 or Nfs1Δ1-55/Isd11 and Isd11, MOCS3-RLD or Uba4 by SPR measurements.**

| Immobilized protein\(^a\) | Protein partner\(^c\) | \(K_D\)\(^d\) | \(\chi^2\) |
|---------------------------|----------------------|------------|----------|
| Isd11                     | Nfs1Δ1-55            | 100        | 0.45     |
|                           | Nfs1Δ1-55/Isd11      | ND\(^e\)  | -        |
| MOCS3-RLD                 | Nfs1Δ1-55            | 86         | 20.8     |
|                           | Nfs1Δ1-55/Isd11      | 480        | 8.0      |
| Uba4                      | Nfs1Δ1-55            | 84         | 14.2     |
|                           | Nfs1Δ1-55/Isd11      | 610        | 7.6      |
| BSA                       | Nfs1Δ1-55            | ND\(^e\)  | -        |
|                           | Nfs1Δ1-55/Isd11      | ND\(^e\)  | -        |

\(^a\) Proteins were immobilized via amine coupling (see Experimental Procedures).

\(^b\) RU, resonance units.

\(^c\) Proteins were injected by using the kinject protocol injecting samples in a concentration range of 0.1 - 6.4 \(\mu\)M. Cells were regenerated by injection of 50 mM HCl.

\(^d\) \(K_D\) values were obtained by global fitting procedures for a 1:1 binding.

\(^e\) ND, no binding detectable.
Figure 1
Figure 3
Figure 4
Figure 6
A novel role for human Nfs1 in the cytoplasm: Nfs1 acts as a sulfur donor for MOCS3, a protein involved in molybdenum cofactor biosynthesis
Zvonimir Marelja, Walter Stöcklein, Manfred Nimtz and Silke Leimkühler

J. Biol. Chem. published online July 23, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M804064200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2008/07/23/M804064200.DC1