THE DUAL ROLE OF THE MOLYBDENUM COFACTOR BIOSYNTHESIS PROTEIN MOCS3 IN tRNA THIOLATION AND MOLYBDENUM COFACTOR BIOSYNTHESIS IN HUMANS

Mita Mullick Chowdhury*, Carsten Dosche†, Hans-Gerd Löhmannsröben‡ and Silke Leimkühler# †

Institute of Biochemistry and Biology, Department of Molecular Enzymology, and †Institute of Chemistry, Department of Physical Chemistry, University of Potsdam, Potsdam, Germany.

1Present address: Institute of Pure and Applied Chemistry, University of Oldenburg, Oldenburg, Germany.

Running Title: The interaction of MOCS3 with MOCS2A and URM1.

†To whom correspondence should be addressed: Tel.: +49-331-977-5603; Fax: +49-331-977-5128; Email: sleim@uni-potsdam.de

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Background: E1-like proteins are required for activation and thiocarboxylation of β-grasp fold proteins involved in sulfurtransfer to cofactors and tRNA.

Results: MOCS3 interacts with both URM1 and MOCS2A in vivo and in vitro.

Conclusion: Molybdenum cofactor biosynthesis and tRNA thiolation steps are linked by the MOCS3 protein in humans.

Significance: To understand the mechanism of protein conjugation and thiocarboxylate formation in sulfurtransfer pathways.

We studied two pathways which involve the transfer of persulfide sulfur in humans, molybdenum cofactor biosynthesis and tRNA thiolation. Investigations using human cells showed that the two-domain protein MOCS3 is shared between both pathways. MOCS3 has a N-terminal adenylation domain and a C-terminal rhodanese-like domain. We showed that MOCS3 activates both MOCS2A and URM1 by adenylation and a subsequent sulfur transfer step for the formation of the thiocarboxylate group at the C-terminus of each protein. MOCS2A and URM1 are β-grasp fold proteins which contain a highly conserved C-terminal double glycine motif. The role of the terminal glycine of MOCS2A and URM1 was examined for the interaction and the cellular localization with MOCS3. Deletion of the C-terminal glycine of either MOCS2A or URM1 resulted in a loss of interaction with MOCS3. ECFP and EYFP fusions of the proteins were constructed and the fluorescence resonance energy transfer efficiency was determined by the decrease in the donor lifetime. The cellular localization results showed that extension of the C-terminus with an additional glycine of MOCS2A and URM1 altered the localization of MOCS3 from the cytosol to the nucleus.

Ubiquitin (Ub) and ubiquitin-like proteins (Ubls) are involved in a large number of diverse processes within the eukaryotic cell. The regulation is achieved by the covalent conjugation of the Ubls to targets proteins via a lysine residue. Only recently, it was discovered that Ubls are not restricted to eukaryotes, with the identification of Pup in bacteria (1) and the characterization of SAMP proteins in archaea (2,3).

Ub-like protein conjugation is dependent on the activation of the C-terminal Gly-Gly motif in an ATP-dependent process, facilitating the formation of an acyl-adenylate and the subsequent thioester formation on a conserved cysteine residue of the activating enzyme (E1) and the Ubl. A cascade of enzymes (E2, E3) is further involved in transesterification reactions for the transfer of the Ubl to a lysine residue of the target protein by formation of an isopeptide bond.

The E1 catalyzed activation of the Ubl resembles the second step of the molybdenum cofactor (Moco) biosynthesis in humans and bacteria. For Moco biosynthesis in humans, the E1-like protein MOCS3 forms a thiocarboxylate group at the C-terminal glycine of the β-grasp fold protein MOCS2A (4-6). Moco is required for the activity of xanthine dehydrogenase, aldehyde oxidase, sulfite oxidase and the mitochondrial amidoxime reducing components, hmARC1 and hmARC2, in humans (7). In Moco, two sulfur atoms of the molybdopterin (MPT) moiety coordinate the molybdenum atom in the final structure (8). Therefore, incorporation of two sulfur atoms in the first intermediate of Moco biosynthesis, cyclic pyranopterin monophosphate (cPMP), is required. The sulfur is mobilized from L-cysteine by NFS1, a pyridoxal-phosphate dependent L-cysteine desulfurase, which forms a persulfide group on its conserved Cys381 residue.
In general, several modified nucleosides containing sulfur atoms were found in tRNA molecules, namely 2-thiocytidine (s₂C), 2-thiouridine (s₂U) derivatives, 4-thiouridine (s₄U) and 2-methylthioadenosine (ms₂A) (15). Especially the 2-thio group of s₂U derivatives is involved in oxidative stress tolerance (19). Urm1 conjugation system. Here, Urm1 conjugates to Ahp1p (alkyl hydroperoxide reductase), a protein involved in oxidative stress tolerance (19). Urm1 has some unique features since it has a dual function in protein conjugation and as a sulfur carrier (20): its structure resembles more those of sulfur carriers, like ThiS (21) and MoaD (22), which are involved in the biosynthesis of sulfur containing cofactors, like Moco and thiamin, and therefore it presents a link between ubiquitin conjugation systems and the evolutionary older cofactor biosynthesis (23).

Only recently, it has been shown that urmylation also occurs in humans and is involved in oxygen stress tolerance (23). It was shown that human URM1 is conjugated to lysine residues of target proteins in its own pathway and that oxidative stress enhances protein urmylation in mammalian cells. However, mechanisms underlying the dual function in protein conjugation and sulfur transfer are still controversially discussed, since on the one hand in cofactor biosynthesis persulfide formation is essential and on the other hand thioster formation is needed for Ub conjugation (12,23).

Therefore, the mechanism of both conjugation and thioconjugation by Ubls to be further elucidated. For such a purpose URM1 displays an excellent example since it is combining both functions. As sulfur carrier, we proposed that MOCS3 connects the two distinct processes in the cell, for Moco biosynthesis and tRNA thiolation, especially since it is the only E1-like activating enzyme that is present in humans for these pathways (5).

In this report, we show that MOCS3 interacts with both URM1 and MOCS2A in human cells. We analyzed the cellular localization of the proteins in addition to their interaction by FRET. The FRET efficiency was calculated by determination of the decrease in the donor lifetime. Since the Gly-Gly motif is the one common sequence feature of the Ubls involved in both acyl-adenylation and thioester or acyl-disulfide formation, the role of the terminal glycine was examined in MOCS2A and URM1, and the effects of the variations on both pathways were compared. In addition, we examined the importance of the C-terminal Gly-Gly motif in terms of its role for the localization of the proteins.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, Media and Growth Conditions.** Cell strains containing expression plasmids were grown aerobically at 30°C in LB medium containing 150 µg/ml ampicillin or 50 µg/ml chloramphenicol. 

_E. coli_ MoaE was expressed in BL21(DE3) cells from plasmid pMWaE15 and purified as described previously by Wuebbens et al. (24). Human MOCS2A and URM1 wild-type proteins were expressed and purified as previously described by Schmitz et al. (5).

**Site-Directed Mutagenesis, Protein Expression, and Purification.** Single amino acid substitutions at the C-termini of the human URM1 or MOCS2A proteins were created using PCR mutagenesis, resulting in URM1-G101A, URM1-G101Δ, and URM1-102G Δ+ or MOCS2A-A88A, MOCS2A-G88A, and MOCS2A-89GΔ variants, respectively. For expression of the variants the URM1 cDNA was cloned into pACYC-duet 1 BglII restriction site and the MOCS2A cDNA was cloned into the sites NdeI and KpnI of pTYB2. _E. coli_ BL21(DE3) cells and ER2566 were used for heterologous expression of URM1 and MOCS2A variants, respectively, as described previously (5).

**Expression of MOCS3.** Sf9 insect cells, derived from _Spodoptera frugiperda_ (Invitrogen), were grown as suspension culture in Sf-900-II SFM medium (Invitrogen, Germany) supplemented with 2% fetal bovine serum (PAN-Biotech, Germany) at 28°C and 180 rpm. For infection with recombinant baculovirus, cells were grown as monolayer cultured at 28°C in a humified incubator.
By PCR the restriction sites BamHI and XbaI were introduced into the MOCS3 cDNA which allowed cloning into the pFastBac vector (Bacto-Bac baculovirus expression system, Invitrogen). The resulting plasmid was designated pMHC30 and expressed MOCS3 as a N-terminal fusion to a His$_6$-tag. Positive recombinant bacmids were used to transfect Sf9 cells using Cellfector II (Invitrogen, Germany) and to produce a baculovirus stock. For protein expression Sf9 cells were infected with high titer viral stock (P2). Cells were harvested by centrifugation 72 h after infection, lysed by sonification and the supernatant was subjected to Ni-NTA chromatography. MOCS3-RLD was expressed and purified as described previously (10,5).

Cell culture techniques.
HeLa and HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, PAN-Biotech, Germany) supplemented with 10% fetal bovine serum (FBS, PAN-Biotech, Germany) and to produce a baculovirus stock. For protein expression Sf9 cells were infected with high titer viral stock (P2). Cells were harvested by centrifugation 72 h after infection, lysed by sonification and the supernatant was subjected to Ni-NTA chromatography. MOCS3-RLD was expressed and purified as described previously (10,5).

Construction of ECFP and EYFP fusion proteins.
Enhanced cyan fluorescence protein (ECFP) and enhanced yellow fluorescence protein (EYFP) is one of the most used FRET pairs (25). Since the donor fluorescence of ECFP is comparably dim, and sensitized emission of the acceptor is hard to detect due to the spectral bleed through of ECFP fluorescence, the fluorescence lifetime of the donor was chosen as parameter to monitor FRET.

MOCS3 cDNA was amplified by PCR and cloned into pECFP-C1 (Clontech) vector using BamHI and Sall restriction sites, resulting in plasmid pZM13. MOCS2A and URM1 variants were cloned using SacI and HindIII restriction sites and ligated as N-terminal fusion proteins into pEYFP-C1 (Clontech) resulting in pMMC2 and pMMC4, respectively.

MPT Synthase Reactions.
MPT synthase reactions were performed at room temperature in a total volume of 400 µL of 100 mM Tris-HCl (pH 7.2). The produced MPT was converted to the stable oxidation product Form A and quantified as described by the published procedures (26,27). The reaction mixtures contained 5 µM of E. coli MoaE, 5 µM MOCS3, 2.5 mM Mg-ATP, 0.6 mM Na$_2$S or sodium thiosulfate and 15 µM of each MOCS2A variant. The reaction was initiated by the addition of 2.5 µM cPMP, which was purified as described previously (28).

Thiosulfate:sulfurtransferase activity of MOCS3.
The sulfurtransferase activity of MOCS3 was measured according to the procedure described by Sörbo (29) in 100 mM Heps (pH 8.0). For determination of the $K_m$ and $k_{cat}$ values, each reaction contained 130 nM MOCS3, 5-500 mM sodium thiosulfate and 0.1 – 50 mM potassium cyanide in a total volume of 500 µL. Thiocyanate was quantified as iron complex by its absorption at 460 nm (ε = 4200 M$^{-1}$cm$^{-1}$).

MOCS3-catalyzed adenylation of MOCS2A and URM1 variants.
For adenylation of MOCS2A and URM1 variants by MOCS3, 20 µM of each protein pair was incubated at 25 °C with 250 µM Mg-ATP, and 2 units of inorganic pyrophosphatase in a total volume of 300 µL. After 90 min, the reaction was stopped by addition of 1% SDS and additional heat inactivation at 95°C for 15 min. To obtain protein-free extracts, samples were transferred to Amicon Ultra concentrators (molecular mass cutoff of 10 kDa, Millipore) and centrifuged at 10,000 g for 15 min. The flow-through was collected and AMP was quantified using the published procedure (27).

In vitro tRNA synthesis.
For quantification of tRNA thiolation by the URM1 variants, the specific tRNA for lysine (UUU) was synthesized in vitro. For synthesis the following primers were used (Fw: 5'-TAATACGACTATAGGCTAGCTAGCTCGGATAGCATAGCTTTAATCTG-3' and Rev: 5'-CGCAGGAACGGGACTTGAACCTGGACGCTCAGGAAGCTACCT-3'). Primers were annealed and ssDNA was synthesized by a reverse transcriptase reaction using the RevertAid™ H Minus Kit (Fermentas). ssDNA was purified using the Extract II KIT (Macherey&Nagel) which served as a template for in vitro transcription using T7 RNA polymerase. Synthesized tRNA was separated by urea containing PAGE. Gel bands corresponding to tRNA were excised and tRNA was dissolved in buffer and concentrated by ethanol precipitation.

In vitro tRNA thiolation and nucleoside analysis.
To quantify the ability of URM1 and the variants to transfer sulfur to the wobble uridine of tRNA$_{Lys}$ (UUU), we incubated 200 µg tRNA$_{Lys}$ (UUU), 5 µM MOCS3, 10 µM URM1, 2.5 mM Mg-ATP and 1 mM sodium sulfide. Additional 10 µM CTU2 was included in the reaction, which was expressed in E. coli with an N-terminal His$_6$-tag and purified by Ni-NTA chromatography and subsequent size exclusion chromatography. The reaction was carried out in a total volume of 200 µL and incubated for 60 min at RT. Afterwards, the thiolated tRNA was precipitated with ethanol and resuspended in 200 µL of 50 mM sodium acetate (pH 5.3) and 20 mM zinc acetate. 3 U of nuclease P1 was added and the mixture was incubated over night at 37 °C. Subsequently, 10 µL of 1 M Tris and 4 U of FastAP (Fermentas) were added and the mixture was incubated for an additional 1 h at 37 °C. Nucleoside analysis was performed as described by Gehrke et al. (30) using a LiChrospher 100 RP 18 column (5 µ, 250 x 4.6 mm).

Surface Plasmon Resonance (SPR) measurements.
All bindings experiments were conducted on a SPR based Biacore$^TM$T200 instrument on CM5 senor chips at a temperature of 25°C and a flow
of 30 µL/min using the control T200 software and evaluation T200 software (GE, Uppsala, Sweden). The autosampler rack containing the samples was cooled throughout the entire measurements to 8°C. Immobilization of proteins yield the following response units (RU) per flow cell: BSA, 698 RU; MOCS3 787 RU. As running buffer, 20 mM phosphate, 150 mM NaCl, 0.005% (v/v) Tween 20, pH 7.4, was used. URM1 and MOCS2A variants with concentrations of 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10 and 20 µM were injected for 4.5 min at a flow rate of 30 µL/min followed by a 15 min dissociation using the kinject command and regeneration of the sensor surface with 50 mM HCl for 1 min. As a control, BSA was used as ligand. Binding curves were corrected by subtractions of buffer injection curves for both flow cells.

Cellular Localization studies and FRET analysis

HEK293 or HeLa cells were grown on poly-L-lysine coated coverslips. Transient transfection of the cells was performed using a modified calcium phosphate method. The DNA-calcium mixture was added drop-wise to the cells and the medium was replaced with fresh culture medium after 8 h. 16-20 h after transfection cells were fixed for 30 min using 4% paraformaldehyde in PBS at 4°C. The cells were washed twice with PBS and mounted onto slides with Mowiol (Roht). Images for subcellular localization were conducted at a Zeiss LSM710 (Zeiss, Jena, Germany) laser scanning confocal microscope equipped with a PlanApo 1.4/63x objective. For fluorescence lifetime imaging microscopy (FLIM), a Visitron Systems imaging system based on an inverted microscope (Axio Observer, Zeiss) equipped with an Infinity 3 confocal scanning head and an additional total internal reflection (TIRF) port was used. As excitation light source a mode locked ps Nd-YAG laser with a repetition rate of 1 kHz was used. The output of the Nd-YAG laser was coupled free space without collimation optics into a 500 µm quartz fibre bundle. This fibre bundle was connected to the TIRF port at the rear side of the microscope via a 355 nm dichroic mirror. Inside the microscope, an arrangement consisting of an additional 355 nm dichroic filter and a 400 nm cut-off filter was used for the separation of excitation and fluorescence emission light. In order to spectrally separate the ECFP and EYFP emissions, a Dual View module (MAG Biosystems) was placed between microscope and detector. This module was equipped with a dichroic filter set for 485 and 540 nm, respectively, with 30 nm bandpass for each channel.

For time gated detection an iCCD camera (Pimax2, Princeton Instruments) was used. For all measurements, 100 frames with a time increment of 0.25 ns and 1.8 ns gate width were acquired to monitor an overall time interval of 25 ns. For each single frame, 250 pulses were integrated on a chip. In order to synchronize the iCCD camera to the laser, the Q-switch monitor output of the laser was used to trigger the TTL input of the CCD control unit. Confocal images were acquired with a CoolSnap HQ2© CCD camera (Photometrics) attached to the Infinity 3 confocal scanning head. Samples were excited with an Ar-Kr ion laser (Innova C70, Coherent) at 456 nm for ECFP or 514 nm for EYFP. Data acquisition and processing were performed with the Metamorph 7.2 software (Molecular Devices). At least 40 cells for each sample were measured. The efficiency of FRET was determined by measuring the donor lifetime of ECFP alone (τD), and in the presence of an acceptor (τDA).

RESULTS

Expression and purification of human MOCS3 from S9 cells.

Earlier attempts to purify the holo MOCS3 protein in an active form failed after expression in heterologous systems like E. coli, Pichia pastoris or S. cerevisiae. Here, an approach was chosen to express human MOCS3 in a baculovirus-insect cell system in S9 cells. The MOCS3 gene was cloned into the pFastBac1 vector resulting in a fusion of the expressed recombinant protein with an N-terminal His6-tag. The S9 cells were grown as suspension cultures transfected at a density of 1 x 10⁶ cells/ml with recombinant virus, and harvested by centrifugation 72 h post infection. The soluble fraction of the cell lysate containing MOCS3 was purified by Ni-NTA chromatography. After elution, one major band was detected on Coomassie Brilliant Blue R stained SDS-polyacrylamide gels with a size of approximately 52 kDa (Figure 1A), corresponding to the calculated molecular mass of 56,695 Da for His₆-tagged MOCS3. The protein was purified with a yield of 1.4 mg/100 ml of S9 cells and a purity of more than 90%.

Analysis of the thiosulfate:sulfurtransfer activity of MOCS3.

Previous studies using the single C-terminal rhodanese-like domain of MOCS3 (MOCS3-RLD) demonstrated that in an in vitro reaction, the protein transfers sulfur from thiosulfate to cyanide (10). The sulfur is bound as a persulfide on Cys412 of the MOCS3-RLD, yielding the following response units (RU) per flow cell. The kinetic constants are listed in Table 1.

<table>
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<tr>
<th>Concentration</th>
<th>kcat (s⁻¹)</th>
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<td>0.16</td>
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<td>1.25</td>
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low catalytic efficiency with thiosulfate as sulfur source shows that thiosulfate is likely not the physiological substrate of MOCS3, as already described by Krepinsky and Leimkühler (31) in a study using the MOCS3-RLD protein. In vivo, the sulfur donor for MOCS3 was proposed to be the L-cysteine-desulfurase NFS1 (11), a protein also involved in FeS cluster biogenesis.

Site directed mutagenesis of MOCS2A and URM1.
The amino acid sequence comparison of URM1 and MOCS2A showed an identity of 24%, with the most striking feature being the C-terminal double glycine motif (Figure 2). To compare the role of the C-terminal Gly-Gly motif of MOCS2A and URM1 for the interaction with MOCS3, the following single-amino acid variants were generated and purified: URM1: G101A, G101Δ, and 102G+ MOCS2A: G88A, G88Δ, and 89G+. The proteins were expressed in a heterologous expression system in *E. coli* and purified by ammonium sulfate precipitation and size exclusion chromatography (URM1) or by Chitin affinity chromatography (MOCS2A). All variants exhibited expression and purification characteristics similar to those of the wild-type proteins, showing that the amino acid changes did not cause significant structural changes that result in protein instability or altered solubility (Figure 1B and 1C). The proteins were purified with a yield of approximately 3 mg/l of expression culture and a purity of at least 95%. For both URM1 and MOCS2A, two bands were obtained after purification (Figure 1B, 1C). By MS/MS peptide mapping we determined that both bands corresponded to URM1 or MOCS2A, respectively, most likely reflecting the presence of the adenylated and non-adenylated species as shown before for *E. coli* MoaD (32).

Analysis of the MOCS3-catalyzed adenylation of the MOCS2A and URM1 variants.
The first step of activation of both MOCS2A and URM1 consists in the formation of an acyl-adenylate group at the C-terminal glycine residue. To test the activity of the N-terminal MoeB-like domain of purified MOCS3, the adenylation efficiency of MOCS3 was analyzed with MOCS2A and URM1 wildtype in addition to the generated protein variants. The incubation mixtures contained MOCS3, Mg-ATP, inorganic pyrophosphatase and either MOCS2A or URM1 variants. The reaction was stopped after varying time points by the addition of 1% SDS and a heat inactivation step. The released AMP was separated on a reversed-phase HPLC column and detected by its absorption at 260 nm. A maximum rate of AMP production (1 mol of AMP/mol of protein) was obtained after 90 min for the wild-type proteins. The results in Figure 3 show that the C-terminal glycine is essential for the adenylation of both MOCS2A and URM1, since a deletion of the last glycine resulted in a complete loss of AMP formation. The variation of the last glycine of both of URM1 and MOCS2A resulted in reduced AMP levels. However, the effect in MOCS2A-G88A was more pronounced with 67% reduction in AMP production in comparison to Urm1-G101A, where the level was only 23% reduced. When an additional glycine was added, the adenylation efficiency in URM1 was reduced to 40% and in MOCS2A to 23%. Overall, the amino acid variations had the same effect on the adenylation efficiency of both proteins, only in MOCS2A the amino acid exchanges had a higher impact on the overall reaction.

Analysis of the rate of MPT formation by MOCS2A.
After formation of the acyl-adenylate on the C-terminus of MOCS2A, sulfur is transferred from the C-terminal MOCS3-RLD and a thiocarboxylate group is formed. In case of MOCS2A, two thiocarboxylated proteins then transfer the sulfur to MOCS2B-bound cPMP and MPT is released. To determine whether the alterations at the C-terminus of MOCS2A affected MPT formation, a defined *in vitro* system was used consisting of purified cPMP, MOCS3, *E. coli* MoaE, Mg-ATP, inorganic pyrophosphatase and MOCS2A. We substituted MOCS2B by *E. coli* MoaE in our assay, which was shown to be more active in vitro (8). As sulfur source either sodium sulfide or thiosulfate were used. Sulfide can be added to activated MOCS2A-AMP without the activity of the C-terminal RLD of MOCS3, while thiosulfate is converted to sulfite and a protein-bound persulfide on MOCS3-RLD, which is then transferred further on to MOCS2A. Like this, the activity of the RLD can be determined and compared to the direct sulfurtransfer with sulfide in vitro. For quantification, produced MPT was converted to its stable oxidation product Form A by oxidation with acidic iodine. The reaction mixtures were separated on a reversed-phase HPLC column, and Form A was quantified by its fluorescence. As shown in Figure 4 (white bars), when sulfide was used as sulfur source, the effect on MPT production of the MOCS2A variants correlated to the adenylation efficiency shown above. The MOCS2A-G88A variant showed a level of 5% MPT produced in the assay in comparison to wildtype MOCS2A. When using the MOCS2A-89G+ and MOCS2A-G88A variant the MPT levels were reduced to 39% or 59%, respectively. When thiosulfate was used as sulfur source, the results obtained were comparable (Figure 4, black bars), however, the overall reaction was less effective and showed a reduction of MPT production of 25% under the assay condition. This might imply that only 75% of the purified MOCS3 has an active RLD. In total, the results are highly consistent with the adenylation level of the MOCS2A variants and show that thiocarboxylate formation and adenylation of MOCS2A correlate.

Quantification of the tRNA thiolation efficiency by URM1. URM1 transfers the sulfur of the
thiocarboxylate group to the wobble uridine 34 of tRNAs, namely tRNA^2-s(UU), tRNA^Glu(UU) and tRNA^Glu(UUC). The introduction of sulfur at the 2-position results in the formation of s^2U or mcm^2s^2U. Prior to the sulfuration reaction, the uridine is proposed to be activated in an ATP-dependent manner by the CTU1-CTU2 complex (33). It was shown previously (16) that in a defined in vitro system using isolated tRNA^2-s(UU), URM1, Uba4 and Ncs6, the yeast homologues of MOCS3 and CTU1, 2-thiouridine was readily formed. The tRNA was digested, nucleosides were separated and formed thionucleosides were quantified. Using this procedure, we analyzed the effects of the alterations of the Gly-Gly motif of URM1 on the rate of 2-thiouridine formation. The results shown in Figure 5 reveal that a drastic reduction of s^2U was observed for the URM1-G101A variant, whereas for the URM1-G101A and URM1-102G+ variants s^2U was detected at a level of 58% and 48%, respectively, in comparison to wildtype URM1 (Figure 5). The results are highly consistent with the adenylation level of the URM1 variants and show that thiocarboxylate formation and adenylation of URM1 correlate.

Analysis of Protein-Protein Interactions by Surface Plasmon Resonance Measurements. To analyze the influence on the C-terminal glycine of MOCS2A and URM1 on the interaction and complex formation with MOCS3 in vitro, SPR measurements were employed for real-time detection of the specific interactions using the purified proteins. Only in the presence of 100 µM ATP in the running buffer, an interaction of MOCS3 with the wildtype MOCS2A or URM1 proteins was determined. K_d values of 0.26 µM for URM1-MOCS3 and 0.47 µM for MOCS2A-MOCS3 were determined using a 1:1 binding model. In case of URM1-G101A, binding curves were monitored, however, since the response did not correspond to a 1:1 binding model, a K_d value could not be calculated. For the other variants no apparent interactions with the immobilized MOCS3 were detectable. The results show that the effects of the variation of the C-terminal glycine of MOCS2A and URM1 variants observed above, are based on an impaired ability of the proteins to form a stable complex with MOCS3. SPR was unable to resolve the differences in the interaction between MOCS3 and alteration of the C-terminus of MOCS2A and URM1, which were detected in the activity assays. Additionally, the results show that the interaction of MOCS2A or URM1 with MOCS3 is more stable in the presence of ATP, as reported before for the Ub-E1 interaction (34).

Analysis of the effects of the MOCS2A and URM1 variants on their cellular localization and the interaction with MOCS3 in human cells. In order to further investigate the role of the C-terminal glycine of MOCS2A and URM1 in vivo, the variants were expressed as an N-terminal EYFP-fusion in HeLa cells. As reported previously (10), our investigations determined that MOCS3 was located in the cytoplasm, whereas MOCS2A showed a dual localization in the cytoplasm and the nucleus (Figure 6A). URM1 showed a similar distribution like MOCS2A, with a localization in the nucleus and in the cytoplasm of HeLa and HEK293 cells (Fig. 6B, data not shown). In the localization studies, we identified a correlation between the length of the Gly-Gly motif and the subcellular localization of the two proteins. When the C-terminal glycine of URM1 was deleted, the overexpressed protein was solely identified in the cytosol (Figure 6B). In addition, the MOCS2A-G88A variant showed lower levels of fluorescence within the nucleus compared to the cytoplasm, which likely corresponds to lower levels in protein amount (Figure 6A). A similar pattern was observed for the expression of MOCS2A-G88A (Figure 6A). In case of the extension of the Gly-Gly motif by one residue, both Ubls exhibited expression like the wildtype proteins, with a localization both in the cytosol and the nucleus. Surprisingly, when MOCS3 was coexpressed with MOCS2A-89G+ or URM1-101G+, MOCS3 was additionally located in the nucleus (Figure 6B), revealing an influence not only on the localization of the Ubls themselves, but in addition also of the activating enzyme.

Further, we also determined the cellular in vivo interaction of MOCS3 with either MOCS2A or URM1 by analyzing their fluorescence resonance energy transfer (FRET). Either N-terminal-tagged ECFP or EYFP variants were used and the FRET efficiency was calculated by determining the decrease in the ECFP donor lifetime. The lifetime was determined by FLIM measurements with an excitation of ECFP at 355 nm and the decay curves were measured in an overall time interval of 25 ns. The decay curves were fitted bi-exponentially with a long lifetime component of τ_1 = τ_0 = 3.9 ± 0.1 ns and a short lifetime of τ_2 = 1.2 ± 0.1 ns in the shorter range, which corresponds to an internal conformational change in ECFP as reported before (35). As positive control, a protein fusion of ECFP and EYFP with peptide linker was used, for which the donor long lifetime component was reduced to τ_1 = τ_0 = 2.8 ± 0.1 ns and the short lifetime of τ_2 = 1.2 ± 0.1 ns remained constant (Figure 7). This corresponds to a decrease in the lifetime of 26%. In comparison the coexpression of ECFP and EYFP alone without the peptide linker did not alter the donor lifetime (Figure 7). The FRET resulting from the coexpression of MOCS3 with wild-type MOCS2A and URM1 resulted in a reduction of 20% in the donor lifetime, due to a higher distance between the fluorophores in the heteromeric complex compared to the distance in the ECFP-EYFP fusion protein. When EYFP was fused to the C-terminus of either URM1 or
MOCS2A, the ECFP-MOCS3 fusion did not change its lifetime, showing that the proteins were unable to interact due to the C-terminal EYFP location.

The resulting lifetimes of the N-terminal EYFP-MOCS2A and URM1 fusions with ECFP-MOCS3 are shown in Figure 7. The results show that replacement of the ultimate glycine of URM1 by alanine resulted in a minor decrease in donor lifetime, whereas deletion or addition of a glycine to URM1 resulted in a drastic decrease in the donor lifetime (Figure 7B). Thus, the proteins were not able to efficiently interact in vivo.

In case of MOCS2A, the alteration of the ultimate glycine in the MOCS2A-G88A variant resulted in a reduced FRET efficiency, whereas the deletion of the ultimate residue in MOCS2A-G88Δ abolished the energy transfer from ECFP completely (Figure 7C). In contrast, the addition of a C-terminal glycine in MOCS2A-89G+, the decrease in lifetime was not as pronounced as for the URM1-102G+ variant. In total, the results are highly consistent with the obtained activities for the protein variants and show that the interaction of both proteins is mainly determined by the C-terminal glycine of the β-grasp fold protein.

DISCUSSION
In this report, we expressed and purified MOCS3 in a baculovirus insect cell system from Sf9 cells, which so far is the only suitable system for the expression of MOCS3. All other attempts to express MOCS3 in eukaryotic systems like P. pastoris or S. cerevisiae did not yield in an active enzyme. Thus, for the first time we were able to demonstrate that MOCS3 was purified in an active form from Sf9 cells, and we demonstrated that both the N-terminal E1/MoeB-like domain and the C-terminal RLD are active. The first indications that MOCS3 might interact with both URM1 and MOCS2A were obtained from in vitro studies in which MOCS3 was replaced by the S. cerevisiae homologue Uba4 (5). The in vitro interaction studies showed, that Uba4 was able to interact with both human MOCS2A and human URM1. In addition to the interaction, these studies were able to demonstrate that a thiocarboxylate group is formed on URM1 in vitro. Later, the thiocarboxylate on Urm1 was confirmed by in vivo studies (23). Even though, S. cerevisiae harbors both URM1-mediated tRNA thiolation and its protein conjugation to Ahp1p, it is not an ideal model system, since the genes for Moco biosynthesis including MOCS2A are absent in this organism. Thus, S. cerevisiae is not a suitable model organism to proof a shared pathway for Moco biosynthesis and tRNA thiolation.

To investigate the role of MOCS3 as a partner for both MOCS2A and URM1 in human cells, we performed FRET studies and investigated the donor lifetime as an indication for the strength of the interaction of the protein-pairs. MOCS3 and either MOCS2A or URM1 were coexpressed as ECFP/EYFP fusion proteins in HeLa cells to determine the FRET between the fluorescent dyes. Due to the high dependence of the distance between the dyes, only the complex formation between MOCS3 and the candidates enable an energy transfer. Our results obtained from the donor lifetime measurements showed that MOCS3 is able to interact with both MOCS2A and URM1 in HeLa cells. The same results were additionally obtained in HEK293 cells (data not shown). Since a similar decay in donor lifetime was obtained, this also revealed a similar distance between the proteins, reflecting similar interaction sites.

For the activation reaction, MOCS3 has to form a heterotetrameric complex with URM1 or MOCS2A, as previously described for Uba4 (5) or the E. coli congeners MoaD and MoeB (4). The crystal structure of the E. coli (MoaD–MoeB)2 complex showed that it is composed of a central MoeB dimer with a MoaD subunit at each end of the dimer. The C-terminus (residues 76–81) is extended into the pocket on the MoeB surface (4) and is therefore accessible for activation. The yeast URM1 crystal structure is very similar to the MoaD structure which has two additional α-helices, as compared to other Ubls (20), and thus is more similar to MoaD in this respect. The structure of yeast URM1 revealed a hydrophobic patch on the surface which is similar to MoaD, and since this region is exposed it might be essential for interaction with MOCS3 (20). Overall, URM1 and MOCS2A share an amino acid sequence identity of 24% (Figure 2). The double glycine motif at their C-termini is the only striking feature in both proteins. To investigate the role of the C-terminal glycine for both proteins for the interaction with MOCS3 in addition to their reactivity, several amino acid variations at the C-terminal glycine were generated. The main purpose was to identify differences between both proteins, since URM1 is a dual function protein and acts in protein conjugation and as a sulfur carrier, and the role of MOCS2A is restricted to act as a sulfur carrier in Moco biosynthesis.

The C-terminal glycine is involved in the first activation step of MOCS2A or URM1 which is catalyzed by MOCS3, involving the formation of an acyl-adenylate as essential prerequisite for subsequent formation of the thiocarboxylate (5). Our results show that the alteration of the terminal glycine motif had the same effects on URM1 and MOCS2A, since all variants showed an impaired rate of adenylation. In addition, also the variation of the length of the glycine motif had similar effects in both Ubls. The deletion of the C-terminal residue totally abolished adenylation, whereas the extension of the motif by one glycine solely reduced the efficiency of the acyl-adenylate formation. The glycine to alanine exchange of the ultimate glycine of URM1 resulted in a slightly impaired ability to form an acyl-adenylate, in contrast the adenylation efficiency for MOCS2A was...
diminished to about a third compared to the wildtype protein.

Considering that the formation of the acyl-adenylate is only the first step of the ATP-dependent activation catalyzed by MOCS3, which is followed by the subsequent thioesterification catalyzed by the RLD, we investigated the sulfurtransfer reaction of thioesterified MOCS2A to cPMP. In our studies MOCS3 was able to thioesterify MOCS2A and the variants using either sulfide or thiosulfate, showing that both domains of MOCS3 are at least 75% active (31). In order to determine the sulfurtransfer rate, MPT formation was monitored. The results showed comparable values as obtained for the rate of adenylation of the proteins, with the MOCS2A-G88Δ variant having the most prominent effect (23). In analogy, we investigated the transfer of sulfur to the wobble uridine of the tRNA^35UTU by URM1 and the variants. In an in vitro system consisting of MOCS3, CTU2, Mg-ATP and sodium sulfide, URM1 was able to catalyze 2-thiouridine formation. The exchange of the C-terminal glycine to alanine and the addition of a glycine decreased the rate of 2-thiouridine formation to 50%, while the deletion of the last glycine had a more severe impact showing only 10% of thiouridine. These results are in good agreement to findings of van der Veen et al. (23) where no thioesterification of the wildtype proteins in MOCS3, as shown in vitro by SPR measurements. For the wild type proteins in presence of ATP Kd values in the nm range were obtained. In comparison for the variants only the URM1-G101D showed an impaired ability to form a complex, which did not obey a 1:1 binding and therefore did not allow the determination of a Kd value. The FRET studies revealed comparable effects in vivo which could be quantified, showing only slightly impaired donor lifetime decrease for the variants compared to the deletion of only the last glycine.

All effects of the variation of the Gly-Gly motif are based on insufficient complex formation with MOCS3, which is followed by the subsequent thioesterification catalyzed by the RLD, we investigated the sulfurtransfer reaction of thioesterified MOCS2A to cPMP. In our studies MOCS3 was able to thioesterify MOCS2A and the variants using either sulfide or thiosulfate, showing that both domains of MOCS3 are at least 75% active (31). In order to determine the sulfurtransfer rate, MPT formation was monitored. The results showed comparable values as obtained for the rate of adenylation of the proteins, with the MOCS2A-G88Δ variant having the most prominent effect (23). In analogy, we investigated the transfer of sulfur to the wobble uridine of the tRNA^35UTU by URM1 and the variants. In an in vitro system consisting of MOCS3, CTU2, Mg-ATP and sodium sulfide, URM1 was able to catalyze 2-thiouridine formation. The exchange of the C-terminal glycine to alanine and the addition of a glycine decreased the rate of 2-thiouridine formation to 50%, while the deletion of the last glycine had a more severe impact showing only 10% of thiouridine. These results are in good agreement to findings of van der Veen et al. (23) where no thioesterification of the wildtype proteins in MOCS3, as shown in vitro by SPR measurements. For the wild type proteins in presence of ATP Kd values in the nm range were obtained. In comparison for the variants only the URM1-G101D showed an impaired ability to form a complex, which did not obey a 1:1 binding and therefore did not allow the determination of a Kd value. The FRET studies revealed comparable effects in vivo which could be quantified, showing only slightly impaired donor lifetime decrease for the variants compared to the deletion of only the last glycine.

Our results are also in good agreement with a previous study investigating the role of the glycine motif of E. coli MoaD (27). These studies together underline the assumption that the ultimate glycine is optimized for the sulfur carrier role for Moco biosynthesis and tRNA thiolation and that especially the length of the tail of the Ubl is critical, since deletion of the tail generally resulted in a inactive protein for all Ubls (27). In contrast, the Ubiquitin-G76A variant exhibited quantitatively acyl-adenylate and thioester formation with its E1 (36), which is consistent with our findings for the URM1-G101A variant, which also showed significant activities in acyl-adenylate formation. This implies that URM1 is more similar to the protein conjugation systems than MOCS2A, which is optimized to serve only as a sulfur carrier protein.

Surprisingly, while determining the FRET efficiency of URM1 and MOCS2A with MOCS3, an influence on the localization by the different variations was observed, revealing a connection between the alteration of the Gly-Gly motif and the subcellular localization. Generally, MOCS3 has a cytosolic localization while URM1 and MOCS2A are both found in the cytosol and the nucleus. The different modifications at the C-terminus of the Ubl effected the protein localization to the same extent. Revealing a restricted localization to the cytosol for MOCS2A-G88A, MOCS2A-G88D and URM1-G101D. In addition, the coexpression of MOCS3 with the variants of URM1 and MOCS2A with the C-terminal elongation of the Gly-Gly motif by one glycine showed not only a nuclear localization, like observed for the wildtype proteins, but also an influence on the localization of their E1 partner, MOCS3, which exhibited an additional localization inside the nucleus. So far, it is unclear whether URM1 or MOCS2A have a specific role in the nucleus. Very recently Van der Veen et al. (23) identified targets of urmylation in humans, among which were besides parts of the tRNA thiolation machinery (CTU1, CTU2) and a deubiquitinating-enzyme (USP15), also proteins involved in nuclear transport like CAS, a protein involved in the shuttling between cytosol and nucleus. Taken into account that upon urmylation, CAS was relocalized to the cytosol and the previous description of the influence of Ubl conjugation on the subcellular localization of proteins (23), there might be an effect on the localization of MOCS3 by its substrates. Van der Veen et al. (23) additionally described the role of the nucleocytoplasmatic shuttling factor CAS as target of urmylation linking the nuclear transport to oxidative stress, which is in good agreement with the observation that nuclear import is halted upon oxidant treatment as also reported by Kodiha et al. (37). Speculation on the influence of urmylation on the arrest of nuclear transport are underlined by similar phenotypes of CAS depletion (38) and URM1 silencing (13), which both result in the arrest in G2/M phase.

On the other hand, the morphological abnormalities caused by reduction of cellular URM1 levels in HeLa cells by shRNA which were shown to be increased cell size and presence of multi nuclei within a single cell, likely also result from translational errors, since the 2-thio modification is implicated in translational fidelity and efficacy as stated by Ikeuchi et al. (39). Additionally, similar findings were also reported for MOCS3 depletion in Caenorhabditis elegans (40). Here, depletion of the MOCS3 homolog reduced the levels of
tRNA thiolation leading to misreading of an opal stop codon and subsequently the suppression of a multivulva phenotype, demonstrating the role of tRNA modification on global cellular processes in this organism. Furthermore, multinuclear cells were also observed after overexpression of URM1. These findings demonstrate an additional role of URM1 for cytokinesis and cell cycle progression. Taken together with the results presented herein a contribution of URM1/MOCS3 to the control of global processes like nuclear transport, cytokinesis and cell cycle progression is possible.

In comparison yeast MDY2, an Ubl-domain protein with no exposed C-terminal Gly-Gly motif, is associated to microtuble-directed nuclear migration, which might have additionally a potential role in these pathways, since MOCS3 was localized in centrosomes where the microtuble emanated. For MDY2 it was described that it is mostly localized in the nucleus, but upon heat stress the protein is relocalized to cytosolic granules. In MYD2 there is no evidence for C-terminal processing, but it is involved in nuclear migration via the microtubulus, which emanates from spindle pole body, the yeast equivalent of the centrosome (41). For SUMO it has been described that it is required for the recruitment of RanGAP1 to kinetochores and the mitotic spindle to control processes during mitosis (42), and has an essential role in the control of nuclear protein import (43). Thus, MOCS3 might be shuttled into the nucleus by either URM1 or MOCS2A. However, whether MOCS3 has a role in the nucleus still has to be investigated.

Our data clearly show that MOCS3 marks the intersection of two pathways, tRNA thiolation and Moco biosynthesis, by activating both URM1 and MOCS2A in human cells (Figure 8). Thus, MOCS3 might be the central switch to determine the faith of the persulfide sulfur at its C-terminal RLD. Since MOCS3 was also shown to be a target for urmylation (23), this might have a regulatory role on the activity of MOCS3 and its interaction with either URM1 or MOCS2A. The influence of urmylation on enzyme activity has to be investigated in future studies.

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REFERENCES

**FIGURE LEGENDS**

**Figure 1:** Purification of MOCS3, MOCS2A, URM1 and their variants. (A) Purification of MOCS3. 4 µg of MOCS3 was separated by 15% SDS-PAGE and stained with Coomassie Brilliant Blue. Purification of URM1 (B) and MOCS2A (C). After purification 3 µg each wildtype protein and the indicated variants were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue.

**Figure 2:** Amino acid sequence alignment of human MOCS2A and human URM1. Identical amino acids are shaded in black.

**Figure 3:** AMP production by the MOCS3. Reaction mixtures contained (A) 20 µM of each URM1 variant or (B) 20 µM of each MOCS2A variant, 250 µM Mg-ATP, 2 units of inorganic pyrophosphatase and 20 µM MOCS3. The reactions were stopped by addition of 1% SDS, and for complete release of AMP, the samples were additionally incubated for 15 min at 95 °C. n.d., not detectable.

**Figure 4:** MPT production by MOCS2A and its variants. MPT was produced using an in vitro system consisting of 5 µM MOCS3, 5 µM MoaE, 1.4 µM cPMP, and 15 µM MOCS2A with either sulfide (white bars) or thiosulfate (black bars) as sulfur source. Reaction mixtures were incubated for 90 min at room temperature and after oxidation of MPT, Form A was quantified by fluorescence detection on a reversed phase HPLC C18 column. For each MOCS2A variant, three independent measurements were performed. n.d., not detectable.

**Figure 5:** 2-Thiouridine formation by URM1 and its variants. 2-Thiouridine formation was determined using an in vitro system consisting of 10 µM CTU2, 5 µM MOCS3, 200 ng of synthesized tRNA^34UU^, 1 mM sodium sulfide, 2.5 mM Mg-ATP and 10 µM of each URM1 variant. Reaction mixtures were incubated 60 min at RT and nucleosides were separated by HPLC after precipitation and digestion of tRNAs. n.d., not detectable.

**Figure 6:** Localization of MOCS3, MOCS2A and URM1 in HeLa cells. (A) Co-localization of ECFP-MOCS3 and EYFP tagged URM1 variants in HeLa cells, and (B) co-localization of ECFP-MOCS3 and EYFP-MOCS2A variants in HeLa cells. HeLa cells were co-transfected with plasmids encoding an N-terminal ECFP-MOCS3 fusion protein and N-terminal tagged EYFP-URM1 or EYFP-MOCS2A. Cells were fixed 24 h post transfection. Localization of MOCS3 and URM1 or MOCS2A variants were determined by confocal microscopy using LSM 710 and the ZEN software for linear unmixing of the two dyes.

**Figure 7:** Quantification of the decrease in donor lifetime. FRET was determined in Hela cells after expression of ECFP and EYFP-tagged proteins by donor lifetime measurement. (A) As controls, coexpression of ECFP and EYFP and an ECFP-EYFP fusion protein were tested. The decrease in donor lifetime of the ECFP-MOCS3 fusion protein in presence of EYFP-tagged variants of URM1 (B) and MOCS2A (C) is shown. For each value at least 40 cells were measured.

**Figure 8:** Model for the interaction of MOCS3 with MOCS2A and URM1 in humans. MOCS3 has an N-terminal MoeB/E1-like domain which adenylates MOCS2A and URM1. The C-terminal rhodanese-like domain (RLD) subsequently forms a thiocarboxylate on both proteins by sulfur transfer. URM1 transfers the sulfur group to uridine 34 on tRNA with the help of the proteins CTU1 and CTU2. MOCS2A forms the active MPT synthase with MOCS2B and transfers the sulfur for the formation of molybdopterin (MPT) in Moco biosynthesis.
Footnotes:

Abbreviations used are: MOCS3, molybdenum cofactor biosynthesis protein 3; Ub, ubiquitin; Ubl, ubiquitin-like modifier; Urm1, ubiquitin-like modifier 1; Moco, Molybdenum cofactor; MPT, molybdopterin; cPMP, cyclic pyranopterin monophosphate; FRET, Förster resonance energy transfer; RLD, rhodanese-like domain; PCR, polymerase chain reaction; Ni-NTA, nickel nitrilotriacetate, RU, response unit (RU), FLIM, fluorescence lifetime imaging microscopy; TIRF, total internal reflection
Table 1: Kinetic parameters of holo MOCS3 and MOCS3-RLD with thiosulfate as sulfur source.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ thiosulfate (mM)</th>
<th>$K_M$ cyanide (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOCS3</td>
<td>2.11 ± 0.20</td>
<td>80.8 ± 3.8</td>
<td>0.50 ± 0.09</td>
</tr>
<tr>
<td>MOCS3-RLD</td>
<td>1.85 ± 0.18</td>
<td>98.5 ± 4.5</td>
<td>0.67 ± 0.11</td>
</tr>
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Apparent $k_{cat}$ and $K_M$ values were determined using the assay described by Sörbo (29) with 5-500 mM sodium thiosulfate and 0.1 – 50 mM potassium cyanide.
Figure 2:

URM1  MAAPLSVEY  EGGGAE LLF DKIKKHRVTL  PGEPEPDIR  NELILIKKNL  LKERPEF  IQ  59
MOC52A  WPLQVEVL  YFAKSAEIT  GVRSETISV  FOS  IILK  AQLWAEIETREPGA  49
Consensus  M  VEV  FAE  G  PQE  L  W  K  P  L

URM1  GDSVRPGILV  LINDADWELL  GELDYQLOLDQ  DSVLFLSTLH  GG  101
MOC52A  DVMQIIF  AVRQ- EYVEL  SDQLLVKPG  DEIAVIPPIS  GG  88
Consensus  VR  I  L  Q  D  I  GG
Figure 3:
Figure 4:
Figure 5:
Figure 6A:
Figure 7:

![Bar chart showing decrease in donor lifetime τₐ (ns) for different constructs.](Image)
Figure 8:
The dual role of the molybdenum cofactor biosynthesis protein MOCS3 in tRNA thiolation and molybdenum cofactor biosynthesis in humans
Mita Mullick Chowdhury, Carsten Dosche, Hans-Gerd Lohmannsroben and Silke Leimkuhler

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