# The Sulfurtransferase Activity of Uba4 Presents a Link between Ubiquitin-like Protein Conjugation and Activation of Sulfur Carrier Proteins†

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ABSTRACT: Because of mechanistic parallels in the activation of ubiquitin and the biosynthesis of several sulfur-containing cofactors, we have characterized the human Urm1 and *Saccharomyces cerevisiae* Uba4 proteins, which are very similar in sequence to MOCS2A and MOCS3, respectively, two proteins essential for the biosynthesis of the molybdenum cofactor (Moco) in humans. Phylogenetic analyses of MOCS3 homologues showed that Uba4 is the MOCS3 homologue in yeast and thus the only remaining protein of the Moco biosynthetic pathway in this organism. Because of the high levels of sequence identity of human MOCS3 and yeast Uba4, we purified Uba4 and characterized the catalytic activity of the protein in detail. We demonstrate that the C-terminal domain of Uba4, like MOCS3, has rhodanese activity and is able to transfer the sulfur from thiosulfate to cyanide in vitro. In addition, we were able to copurify stable heterotetrameric complexes of Uba4 with both human Urm1 and MOCS2A. The N-terminal domain of Uba4 catalyzes the activation of either MOCS2A or Urm1 by formation of an acyl−adenylate bond. After adenylation, persulfurated Uba4 was able to form a thiocarboxylate group at the C-terminal glycine of either Urm1 or MOCS2A. The formation of a thioester intermediate between Uba4 and Urm1 or MOCS2A was not observed. The functional similarities between Uba4 and MOCS3 further demonstrate the evolutionary link between ATP-dependent protein conjugation and ATP-dependent cofactor sulfuration.

Ubiquitin and ubiquitin-like modifiers (Ubls)[1] are involved in diverse biological processes and regulate the activity or function of target proteins through covalent conjugation to them (*1, 2*). They are activated in an ATP-dependent process, which leads to the initial formation of an acyl−adenylate bond between the C-terminus of the modifier and AMP (*3*). The modifier is subsequently transferred onto an active-site cysteine residue in the activating enzyme (E1) via a thioester bond. Subsequently, the Ubl protein is transferred from the E1 enzyme to a conjugating enzyme (E2) in a transthioesterification reaction. The thioester linkages preserve the free energy of ATP and facilitate transfer of the modifier onto the side chain of a lysine residue of a respective target protein via an isopeptide bond, a reaction mediated by E3 proteins.

There are mechanistic parallels between Ubl activation and the biosynthesis of several sulfur-containing enzyme cofactors. In *Escherichia coli*, the sulfur carrier protein MoaD [small subunit of molybdopterin (MPT) synthase], which is involved in molybdenum cofactor (Moco) biosynthesis, is activated in an ATP-dependent manner by MoeB, similar to the activation of Ubls by their cognate E1 enzymes (*4*). However, biochemical and structural studies of the MoeB−MoaD complex revealed that the interaction of MoeB with MoaD resembles only the first step in the activation of ubiquitin (*5, 6*). It was shown that MoeB solely activates the C-terminus of MoaD to form an acyl-adenylate, and no formation of a thioester intermediate could be demonstrated (*6*). Subsequently, the activated MoaD acyl-adenylate is converted to a thiocarboxylate through the action of a sulfurtransferase. In *E. coli*, the sulfur donor is L-cysteine; however, the specific protein involved in the transfer of sulfur to MoaD has not been identified (*7*). The newly formed MoaD thiocarboxylate acts as the direct sulfur donor for the dithiolene group formed during conversion of the sulfur-free precursor Z to MPT, which coordinates the molybdenum atom of Moco (*8*). The activation of bacterial proteins with a ubiquitin fold by adenylation and the subsequent formation of a thiocarboxylate at the C-terminus of these proteins have

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[1] Abbreviations: Ubl, ubiquitin-like modifier; RLD, rhodanese-like domain; MPT, molybdopterin; Moco, molybdenum cofactor; hUrm1, human Urm1; yUrm1, yeast Urm1; TED, triscarboxymethylethylenediamine; MOCS2A-SH, MOCS2A thiocarboxylate; Urm1-SH, Urm1 thiocarboxylate; VE, sedimentation velocity; $f/f_o$, frictional ratio; DTT, dithiothreitol.

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also been described in the pathways for the biosynthesis of cysteine, thioquinolobactin, and thiamin (*9–11*). In the biosynthesis of these sulfur-containing molecules, the thiocarboxylate sulfur of the small sulfur carrier protein is used as direct sulfur donor for the target molecules. Since these bacterial systems share the first adenylation step with the Ubl-dependent protein activiation systems found in eukaryotes, it is believed that the eukaryotic systems are derived from the simpler and universally distributed bacterial systems and have further evolved from those (*4*).

Recent studies have identified the human genes involved in the biosynthesis of Moco (*12*). Human MPT synthase, composed of MOCS2A and MOCS2B, displays homology to its *E. coli* congener, and it was shown that both enzymes exhibit the same mechanism (*13*). Sequence alignments of the human MoeB homologue MOCS3 showed that the N-terminal domain is homologous to *E. coli* MoeB; however, MOCS3 contains an additional C-terminal domain with homologies to rhodaneses (*14*). This rhodanese-like domain (RLD) is present in all eukaryotic homologues, including *Saccharomyces cerevisiae* Uba4, and several bacterial MoeB homologues (*15*).

Rhodaneses (thiosulfate:cyanide sulfurtransferases, EC 2.8.1.1) are widespread enzymes that in vitro catalyze the transfer of a sulfane sulfur atom from thiosulfate to cyanide. They are found not only in combination with other proteins but also as single-domain proteins or as tandem repeats serving as versatile sulfur carriers (*16, 17*). 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 (*16*). MOCS3 was proposed to catalyze both the adenylation and the subsequent generation of a thiocarboxylate group at the C-terminus of MOCS2A during Moco biosynthesis, since it was shown that the rhodanese-like domain of MOCS3 is able to directly transfer the sulfur to MOCS2A via a protein-bound persulfide intermediate (*18*).

Recently, the Urm1−Uba4 protein conjugation system was identified in *S. cerevisiae* (*19*), with Urm1 being 17% identical in amino acid sequence with MOCS2A and Uba4 being 36% identical in amino acid sequence with MOCS3. Uba4 forms a thioester with Urm1, which is covalently attached to the antioxidant protein Ahp1p to modulate its activity in oxidative stress response (*19, 20*). Urm1 and Uba4 are both essential for *S. cerevisiae* budding during vegetative growth, but the function of Urm1 conjugation, also termed urmylation, is only beginning to be understood (*21*). Surprisingly, *S. cerevisiae* is one of the few organisms which does not harbor molybdoenzymes and has only retained the *Uba4* gene encoding a protein similar to MOCS3. In humans, an Urm1 homologue is present in addition to MOCS2A.

Because of the high degree of sequence similarity between MOCS3 and Uba4, it was of interest to investigate the role of Uba4 in detail. We purified and characterized Uba4 and analyzed its role in protein conjugation with human Urm1 (hUrm1) and MOCS2A in vitro. We show that Uba4 forms stable complexes with either MOCS2A or hUrm1 after coexpression in *E. coli*. The C-terminus of Uba4 shows rhodanese activity, and the persulfide group formed on Cys397 is further transferred for the formation of a thiocarboxylate group on MOCS2A and hUrm1. Our results underline the evolutionary relationships between the protein modifiers and sulfur carrier proteins and show that common mechanistic features are retained in Uba4 for both systems.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains, Media, and Growth Conditions.* *E. coli* BL21(DE3) cells, pET16b, pET15b, pET28a, and pACYCduett-1 were obtained from Novagen. Cell strains containing expression plasmids were grown aerobically at either 30 or 16 °C in LB medium containing 150 $\mu$g/mL ampicillin or 50 $\mu$g/mL chloramphenicol.

*Cloning, Expression, and Purification of hUrm1, yUrm1, MOCS2A, S. cerevisiae Uba4, and their Variants.* The gene encoding hUrm1 was amplified from a cDNA bank of human liver cell line HepG2 by PCR. The corresponding PCR product was cloned into the *Nde*I and *Kpn*I sites of the second multiple cloning site of pACYCduett-1, designated pJS26. For expression of MOCS2A, the gene sequence together with the ribosome-binding site was cloned into the *Xba*I and *Bam*HI sites of pET15b, resulting in pSL174. Furthermore, the *Bgl*II and *Bam*HI *MOCS2A* fragment together with the promotor region from pSL174 was cloned into the *Bam*HI site of pACYC184 (*22*), resulting in plasmid pAM32.

Yeast *S. cerevisiae* total chromosomal DNA was used as a template for PCR amplification of the *Urm1* and *Uba4* genes. The gene sequence corresponding to amino acids 38−440 of Uba4 was ligated into the *Nde*I and *Bam*HI sites of pET16b, resulting in pML13, while the full-length y*Urm1* gene was cloned into the *Nco*I and *Bam*HI sites of the pET28a vector (pML14). By using PCR mutagenesis, amino acid exchanges C225A, C397A, and C225A/C397A were introduced into Uba4. For cloning of the rhodanese-like domain of Uba4 (Uba4-RLD), the sequence corresponding to amino acids 291−440 of Uba4 was ligated into the *Nde*I and *Bam*HI sites of pET15b, resulting in pSL240.

For expression of yUrm1, hUrm1, MOCS2A, Uba4, Uba4-RLD, and Uba4 variants, pML14 (yUrm1), pJS26 (hUrm1), pSL174 (MOCS2A), pJS56 (Uba4), pSL240 (Uba4-RLD), pML13 (Uba4-C225A), pJS54 (Uba4-C397A), and pJS55 (Uba4-C225A/C397A) were transformed into *E. coli* BL21(DE3) cells. For expression of Uba4 and variants, cells were grown at 30 °C, and expression was induced at an $A_{600}$ of 0.5 with 300 $\mu$M isopropyl $\beta$-D-thiogalactopyranoside. Cell growth was continued for 16 h at 16 °C, and after cell lysis, the soluble fraction was transferred onto a column with nickel triscarboxymethylethylenediamine (TED, Macheray&Nagel). Uba4 was eluted with 50 mM sodium phosphate, 300 mM sodium chloride, and 250 mM imidazole (pH 8.0) and dialyzed into 50 mM Tris-HCl and 1 mM dithiothreitol (DTT) (pH 9.0). For copurification of Uba4 with yUrm1, hUrm1, or MOCS2A, *E. coli* BL21(DE3) cells were cotransformed with the Uba4 vectors described above and pML14, pJS26, or pAM32. Purification of the protein complexes was achieved by Ni-TED chromatography as described above for Uba4. For purification of yUrm1, hUrm1, and MOCS2A, BL21(DE3) cells were grown at 30 °C, expression was induced at an $OD_{600}$ of 0.5 with 300 $\mu$M isopropyl $\beta$-D-thiogalactopyranoside, and cell growth was continued for 4 h. After cell lysis, MOCS2A or hUrm1 was precipitated by the addition of a 50 to 60% gradient of ammonium sulfate. Final purification of hUrm1 and MOCS2A was achieved by chromatography on a Superose 12 size exclusion column (GE

Healthcare) equilibrated in 100 mM Tris and 200 mM NaCl (pH 7.2). For MPT synthase reactions, the *E. coli* proteins MoaE and MoeB were expressed and purified as described previously (*6, 8*). MOCS3-RLD was expressed as described previously (*14*) and purified using the procedure described above for Uba4.

*MPT Synthase Reactions.* MPT synthase reactions were performed at room temperature in a total volume of 400 $\mu$L of 100 mM Tris (pH 9.0) containing 15 $\mu$M MOCS2A, 2.5 $\mu$M MoaE, 2.5 mM Mg-ATP, and either 10 $\mu$M MoeB, 10 $\mu$M MOCS3-RLD or 10 $\mu$M Uba4/variants using 0.6 mM sodium sulfide or 0.6 mM sodium thiosulfate as the sulfur source. Formation of MPT was started by addition of 2.2 $\mu$M precursor Z, and after specific time points, the reactions were terminated by addition of 50 $\mu$L of acidic iodine. The resulting MPT was oxidized to form A and quantified following the procedure described by Schmitz et al. (*23*).

*Enzyme Assays.* Thiosulfate sulfurtransferase activity was measured according to published procedures (*24, 25*). Using the assay described by Sörbo (*24*), the Uba4 protein was dialyzed into 100 mM Tris-acetate (pH 8.6). For determining $K_m$ and $k_{cat}$ values, each reaction mixture contained 160 nM Uba4, 5−50 mM sodium thiosulfate, and 0.05−3 mM potassium cyanide in a total volume of 500 $\mu$L. Thiocyanate (complexed with iron) was quantified by $A_{460}$ using an $\epsilon$ of 4200 $M^{-1}$ $cm^{-1}$. Using the assay described by Pecci et al. (*25*), reaction mixtures in 1 mL of 50 mM Tris (pH 8.0) contained 2−22.5 mM sodium thiosulfate and 0.125−12.5 mM DTT. The standard assay contained 50 mM sodium thiosulfate and 50 mM DTT. One unit is defined as the amount of enzyme that catalyzes the thiosulfate-dependent oxidation of 1 $\mu$mol of DTT/min.

*Sedimentation Velocity Analytical Ultracentrifugation.* To determine the oligomeric state of Uba4 and Uba4−Urm1 or Uba4−MOCS2A complexes, purified proteins were analyzed by analytical ultracentrifugation. Sedimentation velocity (SV) experiments were conducted in a Beckman Optima XL-I analytical ultracentrifuge (Beckman Couter, Fullerton, CA), using an eight-hole An-50 Ti rotor at 40000 rpm and 20 °C, with 400 $\mu$L samples in standard double-sector charcoal-filled Epon centerpieces equipped with sapphire windows. Data were collected in continuous mode at a step size of 0.003 cm without averaging using either absorption optical detection at a wavelength of 280 nm or interference optical detection. SEDNTERP (www.jphilo.mailway.com) was used to estimate the partial specific volume of the proteins as well as the density and viscosity of the buffer solutions. Prior to centrifugation, proteins were dialyzed against 50 mM Tris and 200 mM NaCl (pH 9.0) in the presence or absence of 0.5 mM DTT. Data were analyzed using the $c(s)$ continuous distribution of Lamm equation solutions (*26*) with SEDFIT (www.analyticalultracentrifugation.com). The $c(s)$ analysis was performed with regularization at a confidence level of 0.95 and a floating frictional ratio ($f/f_o$), time-independent noise, baseline, and meniscus position. The $c(s)$ distribution was transformed to the $c(M)$ distribution.

*Uba4-Catalyzed Adenylation of MOCS2A and hUrm1.* For adenylation of MOCS2A and hUrm1 by Uba4, each MOCS2A−Uba4 or Urm1−Uba4 complex at 25 $\mu$M was incubated with 250 $\mu$M $MgCl_2$, 250 $\mu$M ATP, and 2 units of inorganic pyrophosphatase in a total volume of 300 $\mu$L at 25 °C. After specific time points, the proteins were denatured by incubation at 95 °C for 15 min, and after the addition of 1% SDS, the samples were transferred to Microcon concentrators (molecular mass cutoff of 10 kDa, Millipore) and centrifuged at 10000*g* for 1 h to obtain protein-free extracts. The flow-through was collected, and AMP produced during the reaction was separated by HPLC using a C18 reversed phase column (4.6 mm × 250 mm, ODS Hypersil column, particle size of 5 $\mu$m) equilibrated with 50 mM diammonium phosphate (pH 2.5) and 2% methanol at an isocratic flow rate of 1 mL/min. AMP was quantified relative to an AMP standard solution.

*Electrospray Ionization Mass Spectrometry.* Protein aliquots (1−3 $\mu$L) dissolved in 5 mM ammonium acetate buffer were diluted 1:1 with methanol followed by addition of 10% formic acid (final concentration of 1−10 pmol/$\mu$L). A voltage of approximately 1000 V was applied to the capillary, and ions were subjected to ESIMS/MS on a QTOF 2 mass spectrometer (Micromass, Manchester, U.K.) equipped with a nanospray ion source. For MS/MS analysis, parent ions were selected by the quadrupole mass filter and subjected to collision-induced dissociation. The resulting daughter ions were separated by the TOF analyzer.

*Analysis of Thioester Formation by Nonreducing SDS−PAGE.* For thioester formation, incubations in a 50 $\mu$L final volume of 50 mM Tris (pH 7.5), 2 mM ATP, and 10 mM $MgCl_2$ contained either 12.5 $\mu$M Uba4 or 2.5 $\mu$M yeast UBE1 (Boston Biochem) together with 25 $\mu$M yUrm1, hUrm1, MOCS2A, or human ubiquitin (Boston Biochem). After 1 min at 37 °C, the reaction was quenched by the addition of standard SDS sample buffer from which 2-mercaptoethanol had been omitted. The samples were further incubated on ice for 5 min, before they were subjected to SDS−PAGE at 4 °C in 8.5% polyacrylamide gels for UBE1 or 10% polyacrylamide gels for Uba4.

## RESULTS

*Purification and Characterization of S. cerevisiae Uba4.* For purification, the *Uba4* gene encoding amino acids 38−440 was cloned into the *E. coli* pET16b expression vector, resulting in an N-terminal $His_{10}$-tagged recombinant protein. The soluble fraction of Uba4 was purified by Ni-TED chromatography, and after elution, one major band was visible on Coomassie Brilliant Blue R-stained SDS−polyacrylamide gels with a size of 47 kDa (Figure 1), corresponding closely to the calculated molecular mass of 47493 Da for $His_{10}$-tagged Uba4 (38−440). In contrast, the full-length Uba4 protein could not be purified since the majority of the protein was unstable or existed in inclusion bodies after expression in *E. coli*. Since two highly conserved C*XX*C motifs identified as being involved in Zn binding in *E. coli* MoeB are also present in Uba4, the Zn content of the purified protein was quantified by ICP-OES analysis with a Perkin-Elmer Optima 2100 DV instrument and showed a saturation of 61.3% per monomer (data not shown). To determine the rhodanese activity of Uba4 and for comparative studies, C-terminal amino acids 291−440 of Uba4, corresponding to the C-terminal rhodanese-like domain of Uba4 (Uba4-RLD), were also expressed from the *E. coli* pET15b expression vector, resulting in a $His_6$-tagged recombinant protein. The soluble fraction of Uba4-RLD was purified by Ni-TED chromatography, and after elution, one major band

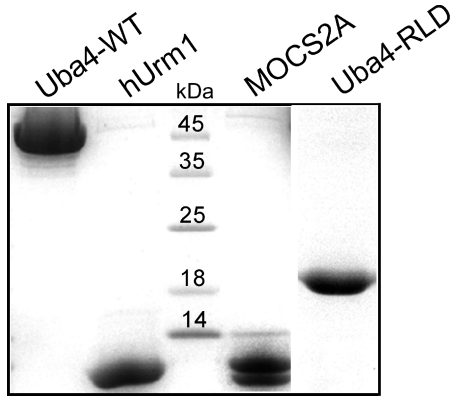


FIGURE 1: Purification and assembly of Uba4, Uba4-RLD, MOCS2A, and hUrm1. Uba4-WT, MOCS2A, hUrm1, and Uba4-RLD (3−5 nmol) were separated by 15% SDS−PAGE and stained with Coomassie Brilliant Blue. The double band obtained after purification of MOCS2A corresponds to the adenylated and carboxylated forms of MOCS2A, as previously reported for MoaD (*6*). Mass spectrometric analyses detected both bands as MOCS2A since the acyl-adenylate group is hydrolyzed under acidic conditions.

was visible on Coomassie Brilliant Blue R-stained SDS−polyacrylamide gels with a size of 20 kDa (Figure 1), corresponding closely to the calculated molecular mass of 20487 Da for $His_6$-tagged Uba4-RLD. The elution position of Uba4-RLD from a size exclusion column revealed that the purified protein existed as a monomer in solution (data not shown), like the homologous MOCS3-RLD protein (*14*).

*Coexpression and Purification of Uba4 with hUrm1 and MOCS2A.* To investigate whether Uba4 can perform similar functions in comparison to the highly homologous MOCS3 protein, we analyzed the ability of Uba4 to interact with either hUrm1 or MOCS2A. For coexpression of $His_{10}$-Uba4 with hUrm1 and MOCS2A in *E. coli* cells, the coding sequences of hUrm1 and MOCS2A were cloned into an *E. coli* expression vector with a P15A origin. The soluble fraction after cell lysis was purified by Ni-TED chromatography, and either the $His_{10}$-Uba4−hUrm1 or the $His_{10}$-Uba4−MOCS2A complex was copurified.

To determine the oligomeric state of purified Uba4 in addition to those of the copurified Uba4−hUrm1 and Uba4−MOCS2A complexes, the proteins were subjected to analytical ultracentrifugation sedimentation velocity (SV) analysis. This method can resolve multiple species in solution and characterize their sizes, shapes, and compositions. First, purified Uba4 was analyzed over a concentration range of 6−21 $\mu$M (Figure 2). The data were best described by a sedimentation coefficient distribution containing two hydrodynamically distinct species with apparent sedimentation values of 3.2 and 4.9 S. The relative ratio of the 4.9 S species to the 3.2 S species increased with increasing loading concentration (Figure 2), indicative of a reversible monomer−dimer equilibrium. Furthermore, an observed shift in the peak positions at different loading concentrations (Figure 2) typically reflects a reversible interaction on the time scale of sedimentation. The estimated apparent molar masses of 46 kDa for the 3.2 S species and 88 kDa for the 4.9 S species are in reasonable agreement with the theoretical molar masses calculated from the amino acid composition for a monomer (47 kDa) and a dimer (94 kDa), respectively, although the maxima in the sedimentation coefficient distribution do not necessarily correspond to the precise values of the actual

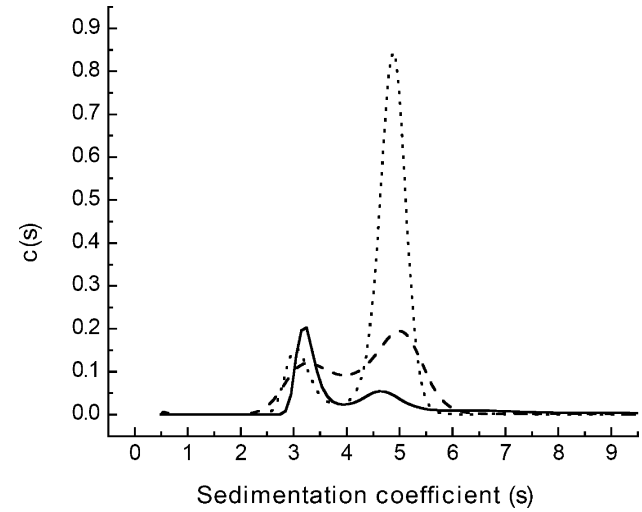


FIGURE 2: Sedimentation velocity analysis of Uba4. Calculated $c(s)$ distributions for Uba4 solutions at different loading concentrations [(—) 6, (---) 14, and (•••) 21 $\mu$M] after maximum entropy regularization are plotted vs sedimentation coefficient $s$ at a $p = 0.95$ confidence level. Experiments were conducted in 50 mM Tris-HCl, 200 mM NaCl (pH 9), and 0.5 mM DTT at 20 °C and a rotor speed of 40000 rpm. Sedimentation was monitored by absorption detection at 280 nm.

sedimentation coefficients of each species, because the positions of these maxima are sensitive to the rate of monomer−dimer equilibrium. The $c(s)$ protocol employed here fits a single frictional coefficient ratio ($f/f_o$) for the entire range of sedimentation values considered; compact, globular proteins typically have a value of 1.2 for $f/f_o$. In the Uba4 experiments, $f/f_o$ was 1.35, which indicates that the protein has a moderately elongated structure in solution.

In addition to the copurified Uba4−MOCS2A and Uba4−hUrm1 complexes, purified MOCS2A and hUrm1 were also analyzed (Figure 1). The sedimentation coefficient profile for purified MOCS2A and hUrm1 contained a single sedimentation coefficient distribution with apparent sedimentation values of 1.3 S for MOCS2A and 1.2 S for hUrm1 (Figure 3). The apparent estimated masses of 9 kDa for MOCS2A and 11 kDa for hUrm1 agree well with the calculated molar masses of monomeric MOCS2A (10 kDa) and monomeric hUrm1 (11 kDa), respectively.

SV analysis of copurified Uba4−MOCS2A and Uba4−hUrm1 protein complexes revealed that in the presence of MOCS2A or hUrm1, the observed dimeric Uba4 species at 4.9 S is shifted to a faster sedimenting species at 5.5 S for the Uba4−MOCS2A complex and 5.6 S for the Uba4−hUrm1 complex (Figure 3). The apparent estimated molar masses of these complexes of 117 and 113 kDa, respectively, corresponded well to the expected molecular masses of a heterotetrameric $(Uba4-MOCS2A)_2$ protein complex (114 kDa) and a heterotetrameric $(Uba4-hUrm1)_2$ protein complex (116 kDa).

*Analysis of the Sulfurtransferase Activity of Uba4.* Previous studies using the C-terminal rhodanese-like domain of MOCS3 (MOCS3-RLD) showed that Cys412, the first residue of a conserved six-amino acid active-site loop, is essential for thiosulfate sulfurtransferase activity since it forms a persulfide group during catalysis (*14, 18*). Cys412 of MOCS3 corresponds to Cys397 in Uba4, which is part of the conserved six-amino acid active-site loop

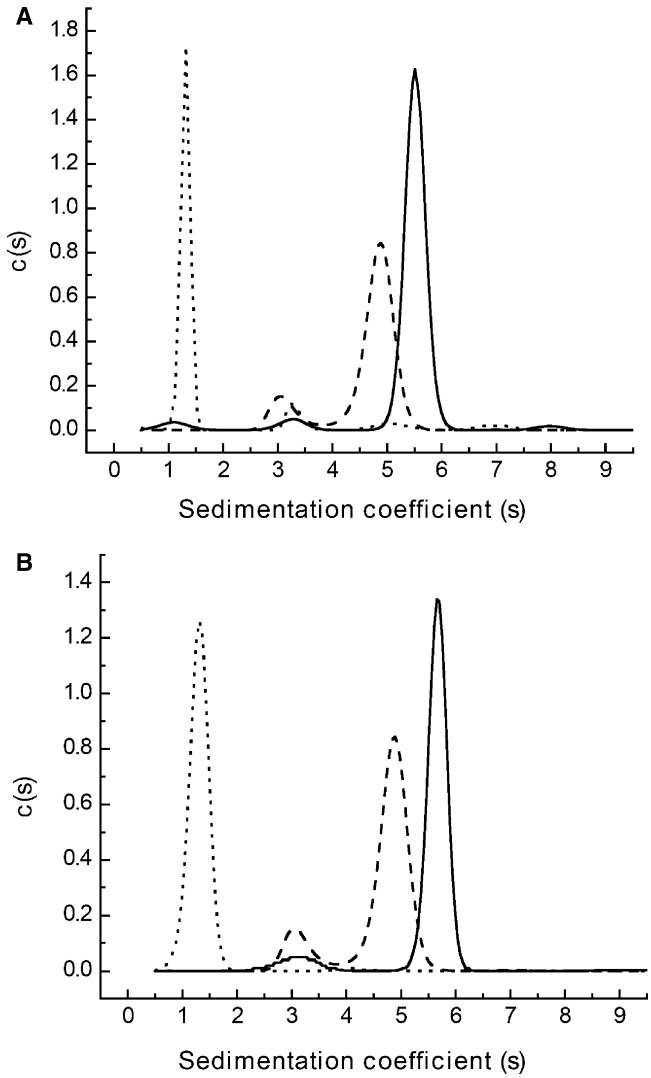


FIGURE 3: Sedimentation velocity analysis of copurified Uba4−MOCS2A and Uba4−hUrml protein complexes. Calculated $c(s)$ distributions with maximum entropy regularization at a $p = 0.95$ confidence level are plotted vs sedimentation coefficient $s$. Experiments were conducted in 50 mM Tris-HCl, 200 mM NaCl (pH 9), and 0.5 mM DTT (Uba4 and Uba4−hUrml) or in the absence of DTT (Uba4−MOCS2A, MOCS2A, and hUrml) at 20 °C and a rotor speed of 40000 rpm. Sedimentation was monitored by absorption detection at 280 nm or by interference detection (MOCS2A). (A) Calculated $c(s)$ distributions for Uba4 [(---) 14 $\mu$M, $ff_o = 1.4$], MOCS2A [(•••) 27 $\mu$M, $ff_o = 1.3$], and the copurified Uba4−MOCS2A complex [(—) 17 $\mu$M, $ff_o = 1.4$]. (B) Calculated $c(s)$ distributions for Uba4 [(---) 14 $\mu$M, $ff_o = 1.4$], hUrml [(•••) 28 $\mu$M, $ff_o = 1.2$], and the copurified Uba4−hUrmI complex [(—) 16 $\mu$M, $ff_o = 1.4$].

($^{397}$CRYGND$^{402}$) of MOCS3-like proteins. The other conserved cysteine residue in Uba4 is Cys225 in the MoeB-like domain, which was previously shown to be essential for protein conjugation of Urm1 (*19*). In vitro, thiosulfate sulfurtransferase activity can be measured either by the method described by Sörbo (*24*) or by that described by Pecci et al. (*25*). The data in Table 1 show that Uba4 catalyzes thiosulfate sulfurtransferase activity using both assays. In comparison to the values obtained previously for MOCS3 (*14*), the $k_{cat}$ value is 165-fold higher for Uba4 ($0.02 \pm 0.003$ $s^{-1}$ for MOCS3) in the Sörbo assay, while higher $K_m$ values were also determined for Uba4 [$K_{m(thiosulfate)} = 0.22$ mM, and $K_{m(cyanide)} = 0.25$ mM for MOCS3] (*15*). For MOCS3, it has previously been reported that the full-length protein (containing an inactive N-terminal MoeB domain) and the MOCS3-RLD protein exhibit the same catalytic activities (*14*). To determine whether the N-terminal MoeB domain has an influence on the sulfurtransferase activity of Uba4, the Uba4-RLD protein (residues 291−420) was separately investigated. As shown in Table 1, the $k_{cat}$ and $K_m$ values for Uba4-RLD were in the same range as those for the full-length Uba4 protein, the exception being that the $K_m$ for thiosulfate was decreased and the $K_m$ for cyanide was increased using the assay described by Sörbo. However, the data show that deletion of the N-terminal MoeB-like domain does not alter the protein stability or drastically reduces the catalytic activity of Uba4. To determine whether Cys397 of Uba4 is the catalytically active cysteine residue, site-directed mutagenesis was performed, to create the Uba4-C397A variant in addition to the Uba4-C225A and Uba4-C225A/C397A variants. While the C225A variant of Uba4 displayed the same kinetic values as the wild-type protein, both Uba4-C397A and Uba4-C225A/C397A variants were completely inactive (Table 1). These results show that Cys397 of Uba4 is essential for thiosulfate sulfurtransferase activity and that Cys225 is not involved in this reaction. In addition, a mass increment of 32 Da as demonstrated by mass spectrometry showed that a persulfide group is formed on residue Cys397 after incubation of the protein with thiosulfate (data not shown). The 32 Da mass increase could not be detected in the Uba4-C397A variant.

Comparison of the thiosulfate sulfurtransferase activities using either DTT (*25*) or $CN^-$ (*24*) as the sulfur acceptor gave similar $k_{cat}$ and $K_{m(thiosulfate)}$ values (Table 1), which was a rather surprising observation, since it has generally been found that DTT inactivates the activity of proteins containing two rhodanese domains (*25*). Thus, usually the rhodanese activity measured as $CNS^-$ production is significantly higher than that measured when $DTT_{ox}$ is formed. The same behavior of Uba4 or Uba4-RLD has been previously observed for MOCS3-RLD (*15*), demonstrating that rhodaneses found linked to other proteins, like MOCS3 and Uba4, are unusual thiosulfate sulfurtransferases which are rather activated by high concentrations of DTT.

*Analysis of the Ability of Uba4 To Form a Thiocarboxylate Group on MOCS2A.* With the finding that Uba4 forms a heterotetrameric complex with MOCS2A, it was of interest to determine whether Uba4, the MOCS3 homologue in yeast, retained its ability to transfer the sulfur from the persulfurated Cys397 further onto the small subunit of MPT synthase by using human MOCS2A. A fully defined in vitro system containing MOCS2A, Mg-ATP, and Uba4 was used to analyze the effects of Uba4 to transfer sulfur to MOCS2A with thiosulfate as the sulfur source. The effective thiocarboxylation of MOCS2A was assayed by its ability to convert precursor Z to MPT in conjunction with *E. coli* MoaE, the large subunit of MPT synthase. We replaced human MOCS2B with *E. coli* MoaE in this assay, since the resulting $(MOCS2A-MoaE)_2$ chimeric MPT synthase was shown to be more efficient in vitro than the $(MOCS2A-MOCS2B)_2$ heterotetramer, due to the higher stability of purified MoaE (*13*). At varying time points, the MPT produced was converted to its stable oxidation product, form A. The reaction mixtures were subjected to HPLC, and form A was quantified by its fluorescence. Figure 4A shows

Table 1: Kinetic Parameters for Uba4 and Variants with Thiosulfate as the Sulfur Source Measured as the Rate of Thiocyanate Formation or as the Rate of Dithiothreitol (DTT) Oxidation

| | thiosulfate:cyanide sulfurtransferase activity[a] | | | DTT:thiosulfate oxidoreductase activity[b] | | |
|---|---|---|---|---|---|---|
| | $k_{cat}$ ($s^{-1}$) | $K_{m(thiosulfate)}$ (mM) | $K_{m(cyanide)}$ (mM) | $k_{cat}$ ($s^{-1}$) | $K_{m(thiosulfate)}$ (mM) | $K_{m(DTT)}$ (mM) |
| Uba4 | 3.3 ± 0.2 | 34.6 ± 4.4 | 0.51 ± 0.01 | 4.6 ± 0.2 | 16.3 ± 0.6 | 2.4 ± 0.1 |
| Uba4-RLD | 1.8 ± 0.5 | 7.4 ± 1.8 | 10.9 ± 3.3 | 2.9 ± 0.2 | 15.3 ± 1.7 | 4.0 ± 1.0 |
| C225A | 3.3 ± 0.3 | 21.4 ± 2.1 | 1.64 ± 0.01 | 3.8 ± 0.2 | 19.6 ± 2.2 | 0.4 ± 0.1 |
| C397A | –[c] | –[c] | –[c] | –[c] | –[c] | –[c] |
| C225A/C397A | –[c] | –[c] | –[c] | –[c] | –[c] | –[c] |

[a] Apparent values determined using the method of Sörbo (*24*). [b] Apparent values determined using the method of Pecci et al. (*26*). [c] Not detectable.

that Uba4 was able to transfer the sulfur from thiosulfate to MOCS2A, as revealed by the increase in form A production over time. A reaction time of 20 min was required to reach maximal activity under the assay conditions. The Uba4-C225A variant exhibited an only slightly reduced initial velocity of MPT production in the assay in comparison to wild-type Uba4, revealing that Cys225 is not essential for the transfer of sulfur to MOCS2A. Replacing thiosulfate with sulfide, which can be directly inserted into MOCS2A without the aid of the Uba4 rhodanese-like domain, resulted in a reduced reaction time in which the maximum was reached after 60 min (Figure 4B), thus demonstrating that the rhodanese domain of Uba4 is quite effective in building the thiocarboxylate group on MOCS2A. Comparison of the effectiveness of Uba4 to sulfurate MOCS2A with the MOCS3 rhodanese-like domain in conjunction with *E. coli* MoeB (*14*) showed that with thiosulfate as the sulfur donor, the ratio of MPT production is drastically reduced (Figure 4A). This result is consistent with the lower $k_{cat}$ values for the rhodanese activity of MOCS3-RLD obtained before and also shows that *E. coli* MoeB cannot substitute completely for the activity of holo-MOCS3. When MOCS3-RLD and thiosulfate are substituted with sodium sulfide in the assay mixture, the velocities of the reaction are comparable to those in the assays containing Uba4, showing that the reaction times for adenylation of MOCS2A by *E. coli* MoeB or Uba4 are comparable (Figure 4B).

Comparison of the efficiencies of Uba4 and the C397A and C225A/C397A variants in producing MPT in the presence of thiosulfate revealed that residue Cys397 is essential for sulfuration of MOCS2A (Figure 4C). Thus, the resulting persulfide group on Cys397 of Uba4 is specifically transferred to MOCS2A during the reaction. In comparison, when using sodium sulfide as the sulfur donor (Figure 4D), similar amounts of MPT were obtained, showing that Cys397 and Cys225 are not essential for the adenylation of MOCS2A (Figure 4D). This result also demonstrates that the adenylation efficiencies of MOCS2A by Uba4 and the Uba4 variants tested are comparable. The slightly different amounts of MPT produced by the Uba4 variant C225A/C397A are likely due to the reduced stability of the protein (Figure S1, Supporting Information).

*Comparison of the Uba4-Catalyzed Adenylation Efficiency of MOCS2A and hUrm1.* Furthermore, it was of interest to determine whether Uba4 catalyzes the formation of the acyl-adenylate group on MOCS2A and hUrm1 at the same rate. To measure the rate of adenylation, Uba4 and Mg-ATP were incubated with either MOCS2A or hUrm1, and after specific time points, the reactions were stopped by addition of 1% SDS and a heat inactivation step. The released AMP was subsequently detected by its absorption at 260 nm after separation by HPLC. A maximum rate of AMP production (1 mol of AMP/mol of protein) was obtained after 30 min for the $(Uba4{-}MOCS2A)_2$ and $(Uba4{-}hUrm1)_2$ protein pairs (Figure 5A). The adenylation of the two Ubl proteins catalyzed by Uba4 was carried out at approximately the same rate. For comparison, the reaction was also analyzed with the Uba4-C225A variant (Figure 5B). Here, the maximum production of AMP (1 mol of AMP/mol of protein) was obtained after 60 min, showing a significantly slower rate in comparison to that of wild-type Uba4. This result is consistent with the previous result measuring the MPT production by Uba4 in conjunction with MOCS2A, showing that the Uba4-C225A variant has a slower rate of MPT production in comparison to that of wild-type Uba4 (Figure 4A). However, we believe that the reduced adenylation

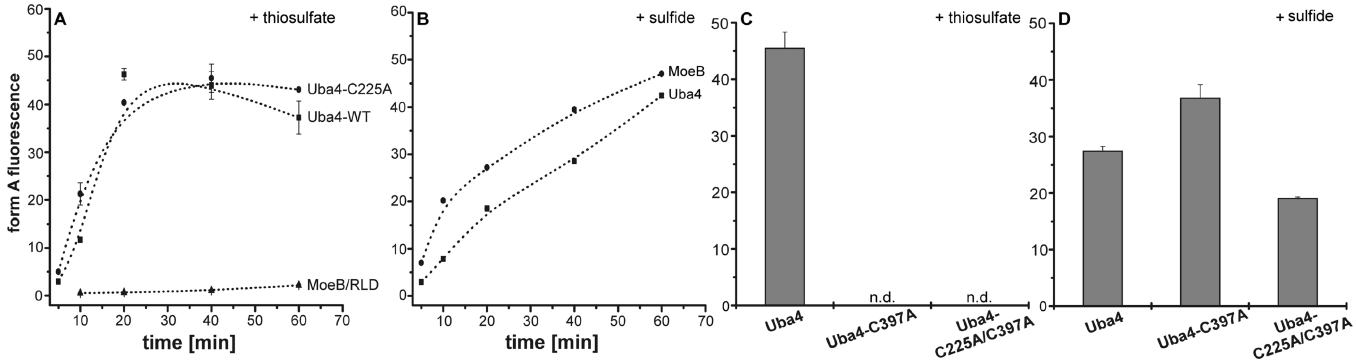


FIGURE 4: MPT production by Uba4 and MOCS2A. Reaction mixtures contained 15 $\mu$M MOCS2A, 2.5 $\mu$M MoaE, 2.5 mM Mg-ATP, and (A) either 10 $\mu$M Uba4 (■), 10 $\mu$M Uba4-C225A (●), or a mixture of 10 $\mu$M MoeB and 10 $\mu$M MOCS3-RLD (▲) incubated with 0.6 mM sodium thiosulfate or (B) either 10 $\mu$M Uba4 (■) or 10 $\mu$M MoeB (●) incubated with 0.6 mM sodium sulfide. The reactions were stopped at the indicated time points. Reactions C and D were terminated after 40 min by the addition of acidic iodine, and the mixtures contained 15 $\mu$M MOCS2A, 2.5 $\mu$M MoaE, 2.5 mM Mg-ATP, and 10 $\mu$M Uba4, Uba4-C397A, or Uba4-C225A/C397A incubated with 0.6 mM sodium thiosulfate (C) or 0.6 mM sodium sulfide (D).

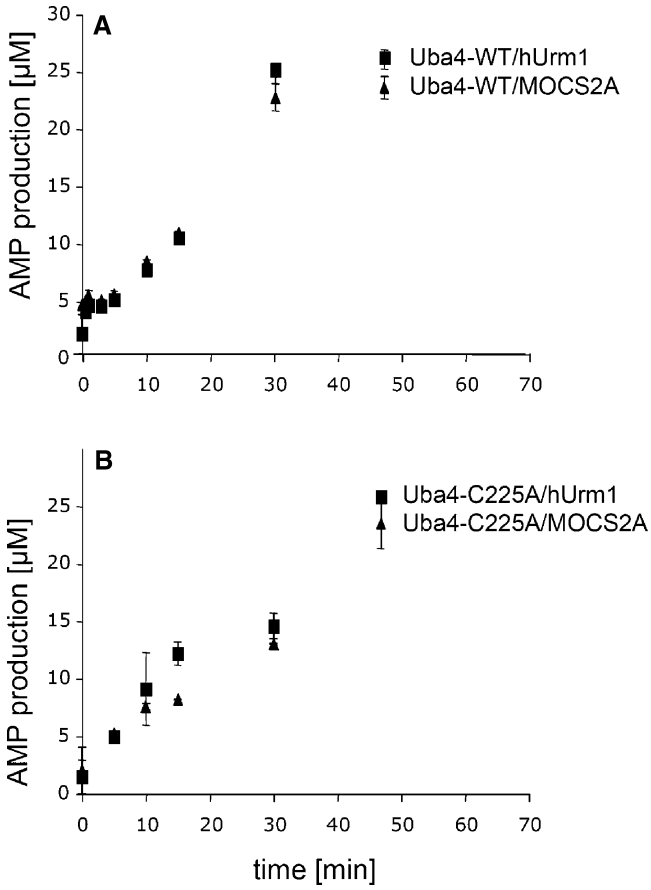


FIGURE 5: AMP production after Uba4-catalyzed adenylation of hUrm1 and MOCS2A. Reaction mixtures contained (A) 25 $\mu$M Uba4 or (B) 25 $\mu$M Uba5-C225A and 250 $\mu$M $MgCl_2$, 250 $\mu$M ATP, 2 units of inorganic pyrophosphatase, and either 25 $\mu$M hUrm1 (■) or 25 $\mu$M MOCS2A (▲). The reactions were stopped at the indicated time points by addition of 1% SDS, and for the complete release of AMP, the samples were additionally incubated for 15 min at 95 °C. A control sample containing 250 $\mu$M ATP was treated in the same manner and subtracted from the incubation mixtures.

efficiency of the Uba4-C225A variant is due to the reduced stability of the protein, as revealed by thermal denaturation studies shown in Figure S1 of the Supporting Information, rather than to the involvement of this residue in the adenylation reaction. AMP production was not obtained with Uba4 or Uba4-C225A in the absence of MOCS2A or hUrm1 (data not shown).

*Electrospray Mass Spectrometry of MOCS2A and Urm1 after Incubation with Uba4, Mg-ATP, and Thiosulfate.* To directly prove that the carboxylate group of MOCS2A is converted to a thiocarboxylate by Uba4, the purified $(Uba4-MOCS2A)_2$ complex was incubated with MgATP, inorganic pyrophosphatase, and sodium thiosulfate before it was subjected to electrospray ionization (ESI) mass spectrometry. As shown in panels A and B of Figure 6, two main species were obtained for MOCS2A with masses of 9755.0 Da for purified MOCS2A (Figure 6A) and 9770.6 Da for MOCS2A in the thiosulfate-treated $(Uba4-MOCS2A)_2$ complex (Figure 6B). The observed 15.6 Da mass difference corresponds to the exchange of an oxygen at the C-terminal MOCS2A carboxylate group with sulfur (+16 Da), presumably forming the MOCS2A thiocarboxylate group. Comparison of the carboxy-terminal fragment ion series obtained by MS/MS of the intact proteins (data not shown) clearly demonstrated that the observed mass increase is located at the C-terminus of MOCS2A.

To determine whether Uba4 is also able to form a C-terminal thiocarboxylate group on hUrm1, the purified Uba4 and hUrm1 proteins were analyzed as described above. As shown in Figure 6D, the same mass increase of 16 Da previously observed for MOCS2A was also observed for hUrm1 (11264.2 Da) in the incubation mixture with Uba4 and thiosulfate (Figure 6D) in contrast to a mass of 11248.3 Da in purified hUrm1 (Figure 6C). MS/MS analyses clearly demonstrated that the observed +15.9 Da mass increase is present at the C-terminus of hUrm1 (data not shown).

To analyze the role of Cys225 and Cys397 of Uba4 in this reaction, Uba4 variants C225A and C397A were also incubated with hUrm1, Mg-ATP, inorganic pyrophosphatase, and sodium thiosulfate and the resulting mass of hUrm1 was determined by ESI-MS analyses. Figure 6E shows that the mixture containing the $(Uba4-C225A-hUrm1)_2$ complex resulted in the +16 Da form of Urm1 (11263.9 Da), while in the $(Uba4-C397A-hUrm1)_2$ complex, hUrm1 showed no mass increase [11248.0 Da (Figure 6F)]. These results demonstrate that Cys225 of Uba4 is not required for the formation of the thiocarboxylate group on hUrm1, while Cys397 of Uba4 is essential for this reaction. To exclude the possibility that Uba4 solely is able to form a thiocarboxylate on the human protein, the same experiments were performed using purified yUrm1. After incubation of separately purified Uba4 with yUrm1, Mg-ATP, inorganic pyrophosphatase, and sodium thiosulfate, yUrm1 revealed a mass increase of 16 Da in comparison to non treated yUrm1 (data not shown). These results clearly show that under the assay conditions used, Uba4 is able to form a thiocarboxylate group at the C-terminal glycine of both Urm1 proteins and MOCS2A.

*Analysis of Formation of a Thioester between Uba4 and hUrm1 or MOCS2A.* Furukawa et al. (*19*) were able to identify a complex between Uba4 and Urm1 after immunoprecipitation from *S. cerevisiae* cells that was sensitive to thiol reagents. This complex was interpreted as a covalent thioester intermediate between Uba4 and Urm1. If Uba4 is an E1-like protein, Uba4 would form a thioester with Urm1 on residue Cys225. However, our investigations of the Uba4-C225A variant showed that this residue is essential neither for thiosulfate sulfurtransferase activity nor for the formation of the thiocarboxylate group on MOCS2A or Urm1. Thus, we reinvestigated the formation of a thioester between Uba4 and hUrm1, yUrm1, or MOCS2A. Mixtures of Uba4 and yUrm1, hUrm1, MOCS2A, and human ubiquitin were analyzed by nonreducing SDS−PAGE. As a positive control, yeast UBE1 was mixed with human ubiquitin and analyzed under the same conditions, in addition to mixtures of UBE1 and hUrm1 or MOCS2A. Figure 7A shows that the complex of UBE1 and human ubiquitin was readily formed as revealed by the peak shift; however, no formation of a thioester complex of any of the other protein mixtures could be detected. In addition, no formation of a thioester was observed between Uba4 and any of the ubiquitin-like proteins, yUrm1, hUrm1, MOCS2A, or ubiquitin (Figure 7B). This result is consistent with previous reports of the homologous MoaD−MoeB pair involved in Moco biosynthesis in *E. coli*, where a thioester intermediate is not formed during the sulfur transfer reaction (*6*). We believe that the

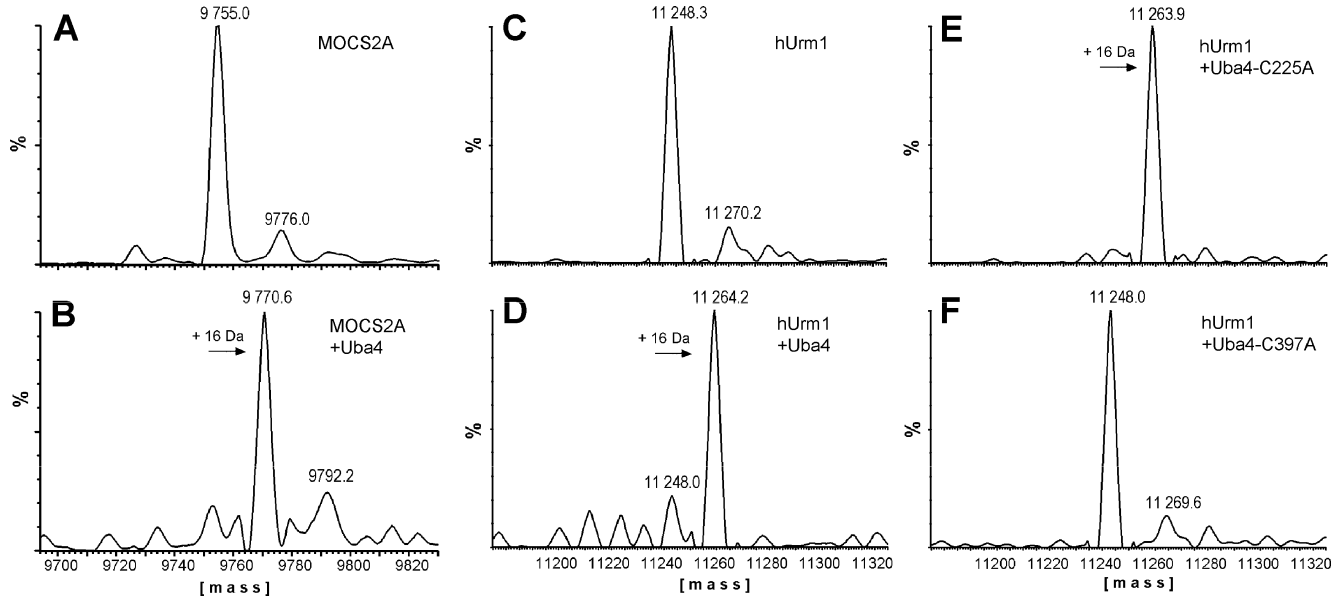


FIGURE 6: Deconvoluted ESI mass spectra of MOCS2A and hUrm1. (A) Purified MOCS2A (9755.0 Da) and (B) the copurified (Uba4−MOCS2A)$_2$ complex incubated with 2.5 mM Mg-ATP, 2.5 mM sodium thiosulfate, and 2 units of inorganic pyrophosphatase. The mass of 9770.6 Da corresponds to thiocarboxylated MOCS2A (+16 Da). (C) Purified hUrm1 (11248.3 Da) and mixtures of (D) purified Uba4 and hUrm1, (E) purified Uba4-C225A and hUrm1, and (F) purified Uba4-C397A and hUrm1 each incubated as described above. The mass of ~11264 Da in panels D and F corresponds to thiocarboxylated hUrm1 (+16 Da). The secondary peaks with mass increases of 22 Da observed in each mass spectrum correspond to the $Na^+$ adduct.

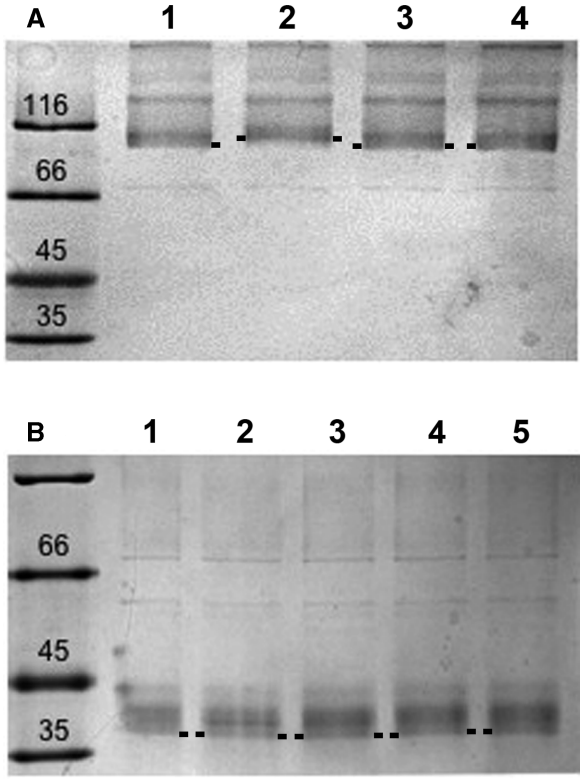


FIGURE 7: Nonreducing SDS−PAGE for the detection of thioester formation. (A) Yeast UBE1 (Boston Biochem) at a concentration of 2.5 $\mu$M was incubated with (1) no addition, (2) 25 $\mu$M human ubiquitin (Boston Biochem), (3) hUrm1, or (4) MOCS2A and separated via 8.5% nonreducing SDS−PAGE. (B) Yeast Uba4 at a concentration of 12.5 $\mu$M was incubated with (1) no addition, (2) 25 $\mu$M yUrm1, (3) hUrm1, (4) MOCS2A, or (5) human ubiquitin (Boston Biochem) and separated via 10% nonreducing SDS−PAGE. Proteins were stained with Coomassie Brilliant Blue.

complex between Uba4 and Urm1 identified by Furukawa et al. (*19*) might be a complex of both proteins which is stabilized by a disulfide bridge.

## DISCUSSION

The structural homologies between the *E. coli* MoaD−MoeB or ThiS−ThiF complexes and the SUMO and NEDD8 activators in complex with their cognate Ubls have demonstrated that the E1-catalyzed activation of Ubl proteins was derived from a step in the more ancient and widespread bacterial systems, including the MoeB-catalyzed activation of MoaD by acyl-adenylation and the subsequent incorporation of sulfur as a thiocarboxylate (*4*). Because of their near-universal presence in all phylogenetic kingdoms, the MoaD or ThiS-like proteins harboring a C-terminal thiocarboxylate are believed to be the evolutionary ancestors of ubiquitin and other Ubls, while the E1 enzymes appear to be derived from MoeB or ThiF-like proteins catalyzing the adenylation of Ubls. Our studies with the *S. cerevisiae* Uba4 protein underline these suggestions and demonstrate that Uba4 is able to perform the activation of two Ubl proteins: MOCS2A (involved in Moco biosynthesis) and Urm1 (involved in protein conjugation). Thus, Uba4 seems to represent the evolutionary link between both systems. Although *S. cerevisiae* is one of the few organisms that does not contain molybdoenzymes, it has retained the MOCS3 homologue Uba4, which contains a C-terminal rhodanese-like domain. Using a reverse-genetic approach, Furukawa and colleagues (*19*) searched in the *S. cerevisiae* genome for potential relatives to *E. coli* MoaD and identified a distantly related protein, which they named Urm1. Utilizing the yeast two-hybrid assay, they identified Uba4 as interacting with Urm1. Prior to this study, it was assumed that Uba4, rather than being a sulfurtransferase, forms only a thioester with Urm1 (*19*). Subsequently, Urm1 is ligated to one or more cellular proteins, one of which has been identified as the antioxidant protein Ahp1p (*20*).

Our studies clearly identify Uba4 as a dimeric protein with thiosulfate sulfurtransferase activity, and site-directed mutagenesis identified Cys397 as the essential amino acid for

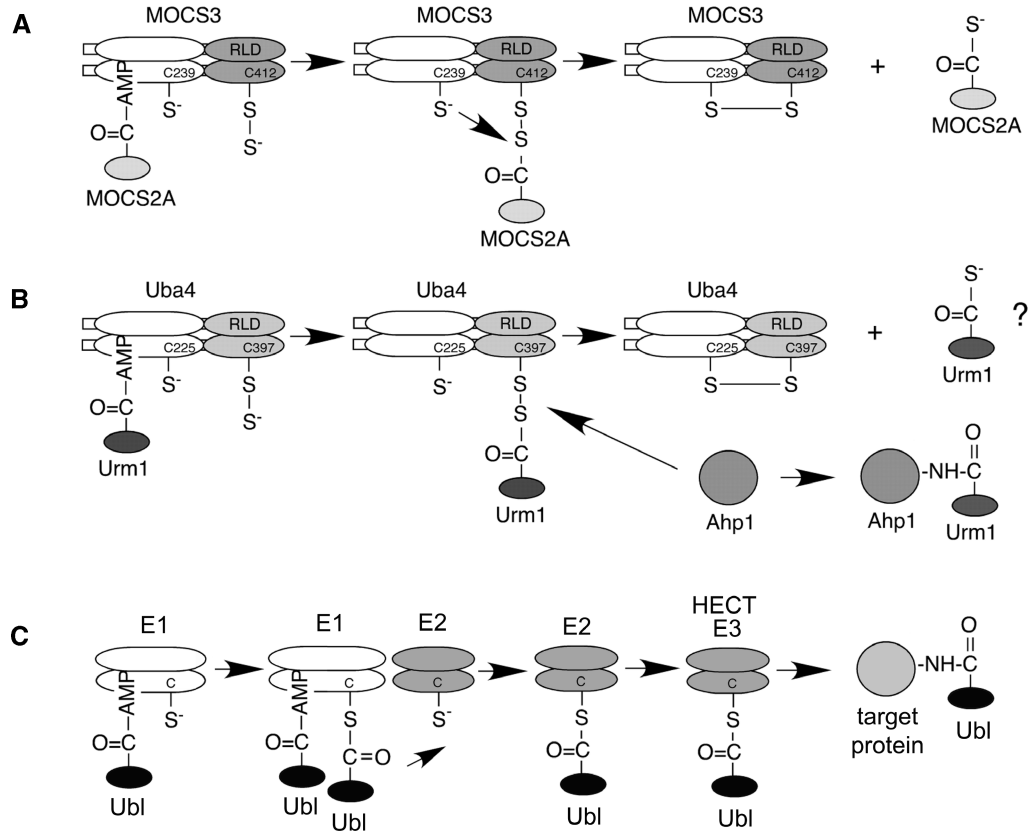


FIGURE 8: Model for the reactions catalyzed by MOCS3 and Uba4. (A) MOCS3 activates MOCS2A by formation of an acyl−adenylate bond. MOCS2A is further transferred to a persulfide group on Cys412 of MOCS3-RLD, forming a disulfide bond. Release of the MOCS2A thiocarboxylate requires a second thiol group, which is proposed to be Cys239, but other reducing factors are also possible. (B) Proposed reaction of Uba4 with Urm1. Uba4 activates Urm1 by formation of an acyl−adenylate bond. Urm1 is further transferred to a persulfide group on Cys397 of Uba-RLD, forming a disulfide bond. Urm1 is either released as thiocarboxylate (hypothetical) or conjugated to target proteins like Ahp1p. It has been shown before that Urm1 is conjugated to target proteins by an isopeptide bond (*20*). (C) Mechanism of transfer of Ubls. Ubls are activated by E1-like proteins, which leads to the initial formation of an acyl-adenylate between the C-terminus of the modifier and AMP. The modifier is subsequently transferred onto an active-site cysteine residue in the activating enzyme (E1) via a thioester bond, and a second Ubl is bound to the AMP site. Subsequently, the Ubl protein is transferred from the E1 enzyme to a conjugating enzyme (E2) and further onto, for example, HECT E3-ligases in a trans-thioesterification reaction. The thioester linkage preserves the free energy of ATP and facilitates transfer of the modifier onto the side chain of a lysine residue of a respective target protein via an isopeptide bond.

this activity. Cys397 corresponds to Cys412 of human MOCS3, on which a persulfide is formed during catalysis that is directly transferred to MOCS2A. Uba4 has a 165-fold higher $k_{cat}$ with thiosulfate than full-length MOCS3 (*14*), or separately purified MOCS3-RLD (*15*). Deletion of the N-terminal MoeB-like domain of Uba4 altered neither the protein stability nor the catalytic activity of the protein, showing that both protein domains act independently and only the C-terminal domain is essential for thiosulfate sulfurtransferase activity. The significance of each amino acid of the six-amino acid active-site loop of MOCS3 was intensively studied by Krepinsky and Leimkühler (*15*), who showed that thiosulfate is not the physiological sulfur donor for MOCS3. Since Uba4 contains an arginine on the second position of the active-site loop instead of a lysine as found in MOCS3, Uba4 in yeast might have adapted to a different sulfur donor than MOCS3 in humans and, thus, has a higher activity with thiosulfate.

In addition to the thiosulfate sulfurtransferase activity of Uba4 using artificial sulfur acceptor molecules, we investigated the ability of Uba4 to transfer the persulfide sulfur formed on Cys397 to other proteins. Uba4 was able to form stable heterotetrameric complexes with both hUrm1 and MOCS2A. We clearly demonstrated that Cys397 of Uba4 is involved in the formation of a thiocarboxylate group of previously adenylated MOCS2A using thiosulfate as the sulfur donor. Site-directed mutagenesis of residues in the MoeB-like domain of Uba4 showed that Cys225 is not essential for the transfer of sulfur to MOCS2A. Surprisingly, Uba4 was also able to form a thiocarboxylate at the C-terminus of hUrm1 and yUrm1 with thiosulfate as the sulfur donor. These results also demonstrated that in our in vitro experiments, Cys225 of Uba4 is also not essential for the formation of the thiocarboxylate group on hUrm1. As for the MoaD−MoeB system, the formation of a thioester intermediate between Uba4 and Urm1 was not identified in our studies. We believe that the thiol-sensitive Urm1−Uba4 covalent complex obtained after immunoprecipitation by Furukawa et al. (*19*) was due to a disulfide bridge rather than a thioester linkage. Thus, a reaction in which adenylated Urm1 is directly transferred to persulfurated Cys397 at the C-terminal RLD to form an acyl-disulfide linkage seems possible (Figure 8). Here, Cys225 of Uba4 might be involved in reductive cleavage of the disulfide bond, as suggested for Cys239 in human MOCS3 (Figure 8). For MOCS3, it was shown that the Cys239 was not required for transfer of sulfur to MOCS2A in vitro; however, in vivo experiments demonstrated a reduced activity of MOCS2A when Cys239 of

MOCS3 was changed to alanine (*14*). Since Furukawa et al. (*19*) showed that Cys225 of Uba4 is essential for protein conjugation of Urm1, we believe that the reductive cleavage of the disulfide bond between Cys397 of Uba4 and Urm1 is catalyzed by Cys225 of Uba4, as shown in the model depicted in Figure 8. This would suggest that Cys225 of Uba4 is important for the reaction without the involvement of a thioester bridge.

The factors dictating whether an acyldisulfide or a thioester is formed during sulfur transfer or protein conjugation are not fully understood at present. It seems that incorporation of sulfur into proteins as thiocarboxylate involves the formation of an acyldisulfide between the Ubl and E1-like protein and that transfer of Ubls for protein conjugation involves thioester formation with E1- and E2/E3-like proteins (Figure 8). Since the involvement of an E2 protein has not been identified for Urm1 conjugation so far, it seems likely that Urm1 is directly transferred from the disulfide intermediate on Uba4-RLD to respective target proteins like Ahp1p (Figure 8). This model is consistent with reports by Kerscher et al. (*3*) and Hochstrasser (*27*) who described that Cys397 of Uba4-RLD is essential for Urm1 protein ligation in yeast; however, details of the reaction were not provided by the authors. Our in vitro studies clearly showed that C397 of Uba4 is essential for sulfur transfer activity of Uba4 through its involvement in the formation of an intramolecular persulfide group, most likely forming an acyldisulfide intermediate with Urm1. Whether thiocarboxylated Urm1 exists in free form in the cell remains to be determined in addition to its role in humans.

Since all eukaryotes contain solely one MOCS3 homologue but usually contain both Urm1 and MOCS2A homologues, we believe that in humans, despite the role of MOCS3 in Moco biosynthesis, which includes the adenylation and sulfuration of MOCS2A, MOCS3 also has the additional role of activating hUrm1 for protein conjugation. So far, nothing is known about the respective targets of hUrm1, since no Ahp1p homologue is present in humans.

Active Moco is essential for the activity of sulfite oxidase, xanthine oxidoreductases, and aldehyde oxidase in humans (*28*). In patients with Moco deficiency, disease-causing mutations were identified in *MOCS1*, *MOCS2*, and gephyrin (*29*), but no mutations in *MOCS3* have been identified to date. 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. We believe that the symptoms caused by the additional loss of function in urmylation of target proteins are even more severe so that mutations in *MOCS3* cause an embryonic-lethal phenotype. In future studies, the exact role of MOCS3 in both pathways in humans needs to be investigated.

In summary, the Uba4−Urm1 system represents the evolutionary link between protein conjugation and protein modification by sulfur carrier proteins, since Urm1 is conjugated to target proteins like Ahp1p and contains a sulfur in the form of a thiocarboxylate group. In analogy, we believe that human MOCS3 after the adenylation reaction forms a thiocarboxylate on MOCS2A or hUrm1. MOCS2A further interacts with MOCS2B and transfers the sulfur to precursor Z for the formation of MPT. The mechanism of transfer of hUrm1 to target proteins must be investigated further, since so far E2 and E3-like proteins were described to be essential for conjugation of Ubls to target proteins. Also, the role of the thiocarboxylate group on Urm1 needs to be further clarified. Our results further support the notion that E1-like proteins in eukaryotes have evolved from MOCS3 and homologues. The C-terminal rhodanese-like domain of MOCS3 is responsible for sulfur transfer, and its function became lost during evolution in E1 enzymes; only the role for protein conjugation was retained and evolved further from that. Unfortunately, so far all attempts to purify holo-MOCS3 in an active form were unsuccessful. However, future studies on MOCS3 and its interaction with hUrm1 will provide more insights into this versatile protein.

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## SUPPORTING INFORMATION AVAILABLE

Circular dichroism (CD) and thermal denaturation studies of Uba4 and its variants. This material is available free of charge via the Internet at http://pubs.acs.org.

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