Molybdenum Cofactor Biosynthesis in Humans: Identification of a Persulfide Group in the Rhodanese-like Domain of MOCS3 by Mass Spectrometry†

Andreas Matthies,‡ Manfred Nimtz,§ and Silke Leimkühler*,‖

Institut für Pflanzenbiologie, Technische Universität Braunschweig, 38023 Braunschweig, Germany, Institut für Strukturbiologie, Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, 38124 Braunschweig, Germany, and Institut für Biologie und Biochemie, Universität Potsdam, 14476 Potsdam, Germany

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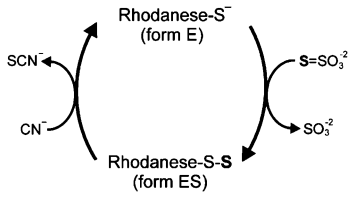
ABSTRACT: The human MOCS3 protein contains an N-terminal domain similar to the *Escherichia coli* MoeB protein and a C-terminal segment displaying similarities to the sulfurtransferase rhodanese. MOCS3 is proposed to catalyze both the adenylation and the subsequent generation of a thiocarboxylate group at the C-terminus of the smaller subunit of molybdopterin (MPT) synthase during Moco biosynthesis in humans. Recent studies have shown that the MOCS3 rhodanese-like domain (MOCS3-RLD) catalyzes the transfer of sulfur from thiosulfate to cyanide and is also able to provide the sulfur for the thiocarboxylation of MOCS2A in a defined in vitro system for the generation of MPT from precursor Z. MOCS3-RLD contains four cysteine residues of which only C412 in the six amino acid active loop is conserved in homologous proteins from other organisms. ESI-MS/MS studies gave direct evidence for the formation of a persulfide group that is exclusively formed on C412. Simultaneous mutagenesis of the remaining three cysteine residues showed that none of them is involved in the sulfur transfer reaction in vitro. A disulfide bridge was identified to be formed between C316 and C324, and possible roles of the three noncatalytic cysteine residues are discussed. By ESI-MS/MS a partially gluconoylated N-terminus of the His_6-tagged MOCS3-RLD was identified (mass increment of 178 Da) which resulted in a heterogeneity of the protein but did not influence sulfurtransferase activity.

Rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) is a widespread enzyme that catalyzes in vitro transfer of a sulfane sulfur atom from thiosulfate to cyanide via a protein-bound persulfide group. Rhodanese-like proteins are either composed of a single catalytic rhodanese domain or fusions of two rhodanese domains, with its C-terminal domain containing the putative catalytic cysteine (*1*). Furthermore, catalytic or inactive rhodanese domains are also found in other proteins such as ThiI (*2*) or in Cdc25 phosphatases (*3*). The best characterized enzyme is bovine liver rhodanese, a member of the two domain family (*4*, *5*). The catalytic mechanism of this enzyme involves a double displacement mechanism that requires the transient formation of a stable persulfide-containing intermediate (ES, Scheme 1).

In bovine rhodanese the transferred persulfide sulfur is bound to a cysteine residue of a six amino acid active site loop that defines the ridge of the catalytic pocket which is expected to play a key role in substrate recognition and catalytic activity (*1*). Site-directed mutagenesis of bovine rhodanese showed that, out of four conserved cysteines, only the cysteine residue of the six amino acid active site loop is required for thiosulfate:sulfurtransferase activity (*6*). Furthermore, the biochemical and structural characterization of *Escherichia coli* GlpE composed of a single rhodanese domain (*7*, *8*) showed that the N-terminal domain as found in bovine rhodanese is not essential for sulfur transfer. The biological role of rhodaneses is still largely debated. Proposed functions include cyanide detoxification (*9*), formation of prosthetic groups in iron−sulfur cluster proteins (*10*), and sulfur transfer for thiamin or thiouridine biosynthesis (*2*, *11*).

Scheme 1



The first physiological role of a rhodanese-like domain in humans was identified in the pathway for the biosynthesis of the molybdenum cofactor (Moco)[1] (*12*). In this pathway, the human MOCS3 protein is a two-domain protein with an N-terminal domain similar to the *E. coli* MoeB protein and a C-terminal segment displaying similarities to rhodaneses. Similar to ubiquitin activating enzymes (E1), the N-terminus of MOCS3 is expected to activate the C-terminal glycine of

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* To whom correspondence should be addressed. Phone: +49 (331) 977-5603. Fax: +49 (331) 977-5419. E-mail: sleim@rz.uni-potsdam.de.

‡ Technische Universität Braunschweig.

§ Gesellschaft für Biotechnologische Forschung.

‖ Universität Potsdam.

[1] Abbreviations: MPT, molybdopterin; Moco, molybdenum cofactor; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; RLD, rhodanese-like domain.

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MOCS2A to form an acyl adenylate. Subsequently, the C-terminal rhodanese-like domain (RLD) of MOCS3 acts as a direct sulfur donor for the formation of a thiocarboxylate group on MOCS2A (*12*). The MOCS2A thiocarboxylate sulfur is used for the generation of the dithiolene moiety of molybdopterin (MPT) which coordinates the molybdenum atom in Moco.

MOCS3-RLD contains four cysteine residues of which only C412 of the six amino acid active loop is conserved with homologous proteins of other organisms. Site-directed mutagenesis showed that exchange of C412 to alanine resulted in loss of activity (*12*), although direct proof of a postulated persulfide formed on C412 remained to be identified.

In this report the separately purified human MOCS3-RLD domain was analyzed in molecular detail by site-directed mutagenesis and mass spectrometry. All His_6-tagged protein forms were found to have lost their N-terminal methionine residue and were shown to bear an N-terminal gluconoyl residue in significant amounts (10−50%) that does not influence catalysis. For the first time ESI-MS/MS was performed on a rhodanese-like protein normally associated with an additional protein domain, providing direct evidence for the formation of the persulfide on C412. Simultaneous mutagenesis of the remaining three nonconserved cysteine residues of MOCS3-RLD showed that they are not persulfurated during catalysis. A disulfide bridge was identified between C316 and C324, which is not essential for sulfur transfer in vitro. However, it remains possible that the disulfide bridge of the two cysteine residues in the linker region between the N-terminal MoeB and the C-terminal RLD domains of MOCS3 is involved in the mechanism of sulfur transfer to form the thiocarboxylate group on MOCS2A in vivo.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma or Duchefa and used as provided. *E. coli* BL21(DE3) cells and pET15b were obtained from Novagen. Thrombin protease was obtained from GE Healthcare. Cell strains containing expression plasmids were grown aerobically at 30 °C in LB medium containing 150 μg/mL ampicillin.

Cloning, Expression, and Purification of MOCS3-RLD Cysteine to Alanine Variants. Amino acid exchanges C316A/C324A and C316A/C324A/C365A were introduced into MOCS3-RLD by PCR mutagenesis, and the corresponding fragments were subsequently cloned into the *Nde*I and *Bam*HI sites of pET15b, resulting in an N-terminal His_6-tag fusion of the MOCS3-RLD variants. For expression of MOCS3-RLD and variants, the corresponding plasmids were transformed into *E. coli* BL21(DE3) cells. All variants were expressed at 30 °C in BL21(DE3) and purified by affinity chromatography and size exclusion chromatography as described previously (*12*). Cleavage of the N-terminal His_6 tag of MOCS3-RLD wild type was carried out with 1 unit of thrombin/mg of protein for 16 h overnight at 23 °C. Separation of the resulting protein and the His_6 tag was performed by gel filtration on a Superose 12 column equilibrated with 50 mM HEPES and 200 mM NaCl, pH 8.0. MOCS2A, MoaE, and MoeB were purified as described previously (*13*, *14*). Protein concentrations were determined by the method of Bradford or by using their calculated molar extinction coefficients at 280 nm.

Preparation of Persulfurated and Persulfide-Free MOCS3-RLD Variants. The persulfide-free form of the protein was prepared by adding 5 mM KCN to an enzyme solution in 50 mM HEPES and 200 mM NaCl, pH 8.0. After incubation of 1 h, the KCN and thiocyanate were removed by gel filtration on a Nick gel filtration column (GE Healthcare) equilibrated in 3 mM NH_4OAc, pH 8.0. Persulfurated protein was prepared by adding 25 mM thiosulfate to an enzyme solution in 50 mM HEPES and 200 mM NaCl, pH 8.0. After incubation of 1 h, thiosulfate was removed by gel filtration on a Nick gel filtration column (GE Healthcare) equilibrated in 3 mM NH_4OAc, pH 8.0.

Treatment of MOCS3-RLD Variants with Iodoacetamide. Iodoacetamide treatment was carried out to protect sulfhydryl groups of MOCS3-RLD from oxidation during mass spectrometric analysis by adding 20 mM iodoacetamide to the enzyme solution in 50 mM HEPES and 200 mM NaCl, pH 8.0. To remove excess iodoacetamide and salt, the solution was gel filtered into 3 mM NH_4OAc, pH 8.0.

DTNB Titrations. The sulfhydryl groups of native and denatured MOCS3-RLD variants were determined according to the method of Ellman (*15*). All assays were performed in 50 mM HEPES and 200 mM NaCl, pH 8.0, in a final volume of 1 mL with protein concentrations of 10 μM. To denature the MOCS3-RLD variants, 0.5 g of solid guanidinium chloride was added to the samples and dissolved to achieve a final concentration of 5 M. To determine sulfhydryl groups, the protein solutions were incubated with 400 μM DTNB for 30 min at room temperature. Control incubations were prepared by adding the same amount of DTNB to the same buffer without protein. The intensely yellow dianion of 5-thio-2-nitrobenzoic acid was quantified by subtracting the A_{412nm} of the control incubations and using a calibration curve with cysteine or an extinction coefficient of 13600 M^{-1} cm^{-1}.

Enzyme Activity Assays. Sulfurtransferase activity was measured by the method of Westley (*16*). Reaction mixtures contained 100 mM Tris−acetate, 50 mM ammonium thiosulfate, and 50 mM KCN, pH 8.6, in a volume of 500 μL. After 5−30 min formaldehyde (250 μL, 15%) was added to quench the reaction, and color was developed by the addition of 750 μL of ferric nitrate reagent [100 g of $Fe(NO_3)_3 \cdot 9H_2O$ and 200 mL of 65% HNO_3 per 1500 mL]. Thiocyanate (complexed with iron) was quantitated by A_{412nm} using $\epsilon =$ 4200 M^{-1} cm^{-1}.

MPT synthase reactions were performed at room temperature in a total volume of 400 μL of 100 mM Tris-HCl, pH 7.2 (*12*). MOCS2A-OH (4 μM), 4 μM MoaE, 4 μM MoeB, 2 μM persulfurated form of MOCS3-RLD variants, 2.5 mM $MgCl_2$, and 2.5 mM ATP were incubated for 15 min on ice, and the formation of MPT was started by addition of 2.3 μM precurzor Z. Purified precursor Z was obtained from cells that contain a mutation in *moaD* and thus accumulate the precursor (*17*). After 2 h the reaction was terminated by the addition of 50 μL of acidic iodine to convert MPT to form A (*18*).

Tryptic Digestion of MOCS3-RLD for MS/MS. Protein solutions were digested by addition of equal amounts of a trypsin (sequencing grade, Promega) solution (2 $\mu g \cdot mL^{-1}$ trypsin in 50 mM NH_4HCO_3) and incubation at 37 °C overnight. The resulting peptides were desalted using C-18

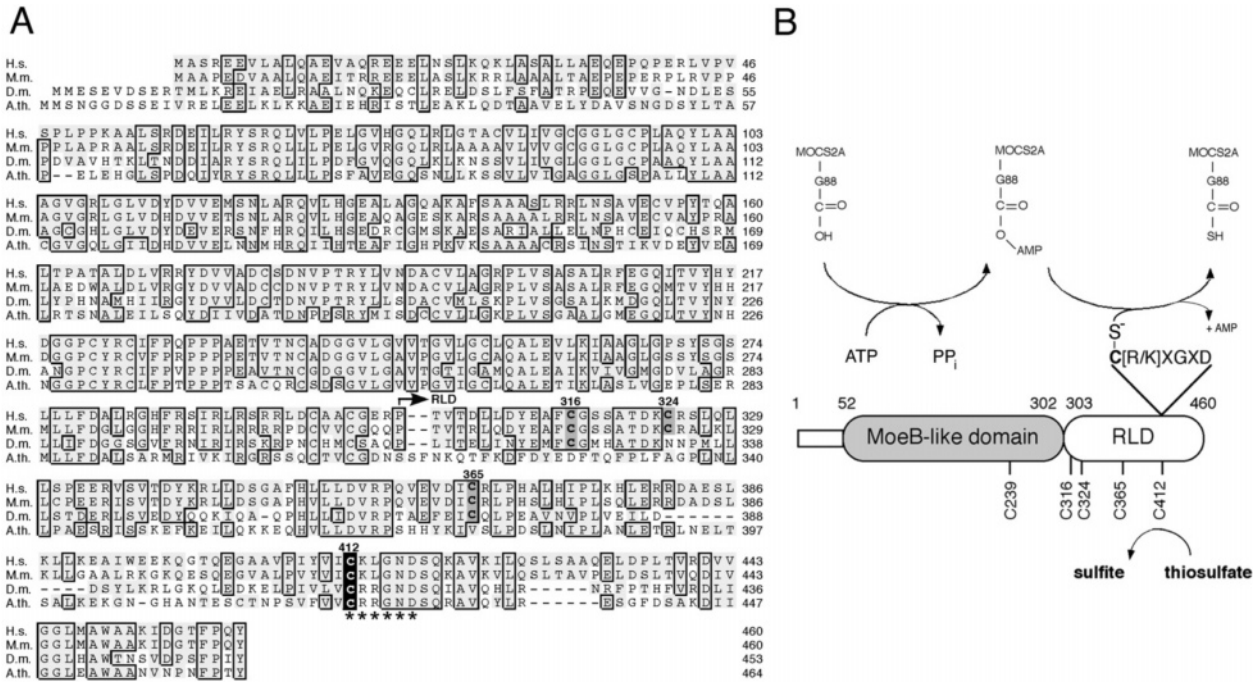

FIGURE 1: Amino acid sequence alignment and domain structure of human MOCS3. (A) Amino acid sequence alignment of human MOCS3 (H.s.) with homologous proteins from *M. musculus* (M.m.), *D. melanogaster* (D.m.), and *A. thaliana* Cnx5 (A.th.). Identical amino acids are boxed, and homologous amino acids are shaded in gray. Highlighted are the cysteine residues found in the RL domain starting at residue 303 of human MOCS3 (arrow) and the six amino acid active site loop (stars). (B) Two-domain structure of MOCS3. The four MOCS3-RLD cysteine residues are indicated. The six amino acid active site loop containing the catalytically active C412 is shown in addition to the reaction catalyzed. The N-terminus of MOCS3 is expected to activate the C-terminal glycine of MOCS2A to form an acyl adenylate. Subsequently, the C-terminal rhodanese-like domain (RLD) of MOCS3 acts as a direct sulfur donor for the formation of a thiocarboxylate group on MOCS2A.

ZipTip pipets (Millipore) according to the manufacturer's instructions. After being washed with 0.1% formic acid, elution of peptides was performed with 10 μL of 0.5% formic acid in 65% methanol.

Electrospray Ionization MS. Aliquots (1−3 μL) of the intact MOCS3-RLD protein variants dissolved in 5 mM NH_4-OAc buffer were diluted 1:1 with methanol followed by addition of 10% formic acid (final concentration 1−10 pmol/μL). In another MS experiment, the desalted peptide mixtures corresponding to 10−50 pmol of protein obtained by tryptic digestion were similarly applied to a nanospray gold-coated glass capillary placed orthogonally in front of the entrance hole of a QTOF-II instrument (Micromass, Manchester, U.K.). Approximately 1000 V was applied to the capillary, and ions were separated by the time-of-flight analyzer. For MS/MS analysis parent ions were selected by the quadrupole mass filter and subjected to collision-induced dissociation. Resulting daughter ions were then separated by the TOF analyzer. Protein spectra were deconvoluted using the MaxEnt1 software package, peptide spectra were further processed by the MaxEnt3 program (Micromass, Manchester, U.K.), and the peptide sequence was determined using the PepSeq software.

RESULTS

ESI-MS of Heterologous Expressed His_6-Tagged MOCS3-RLD. Amino acid sequence alignments of human MOCS3 with homologous proteins from *Arabidopsis thaliana*, *Mus musculus*, and *Drosophila melanogaster* showed that in the C-terminal rhodanese-like domain (RLD) of these proteins only the cysteine residue of the six amino acid active loop is conserved (Figure 1A). Up to four additional cysteine residues are present in this domain in different organisms. In a previous report it was shown that only the exchange of the active site loop cysteine residue C412 of MOCS3-RLD to alanine abolished thiosulfate:sulfurtransferase activity completely, showing the importance of this residue for catalysis (*12*). However, direct proof for the formation of a persulfide group on C412 during the sulfur transfer reaction remained to be identified. In addition, we showed that C239 of the N-terminal MoeB-like domain of MOCS3 is most likely involved in the sulfur transfer mechanism of the persulfide sulfur to MOCS2A (*12*). Since the purification of holo-MOCS3 after heterologous expression in *E. coli* cells resulted in an inactive N-terminus of MOCS3 (*12*), the C-terminal 158 amino acids of the RL domain were purified separately with an N-terminal His_6 tag for further characterization (Figure 1B).

To determine the exact mass of recombinant MOCS3-RLD, electrospray mass spectrometry (ESI-MS) was performed. As shown in Figure 2A, the main species of the protein yielded an average mass of 19756.7 Da. This mass is 134.0 Da lower than the calculated mass of 19890.7 Da of the protein. To determine the position of the modification of the protein sequence, a tandem MS experiment was performed. All assigned N-terminal fragments had a mass 131.2 Da lower than expected, suggesting that the N-terminal methionine introduced by the His_6 tag of pET15b is missing. The remaining mass difference of −2.8 Da between the calculated and measured mass is larger than the expected instrument error, which points to the presence of a disulfide bond in the protein.

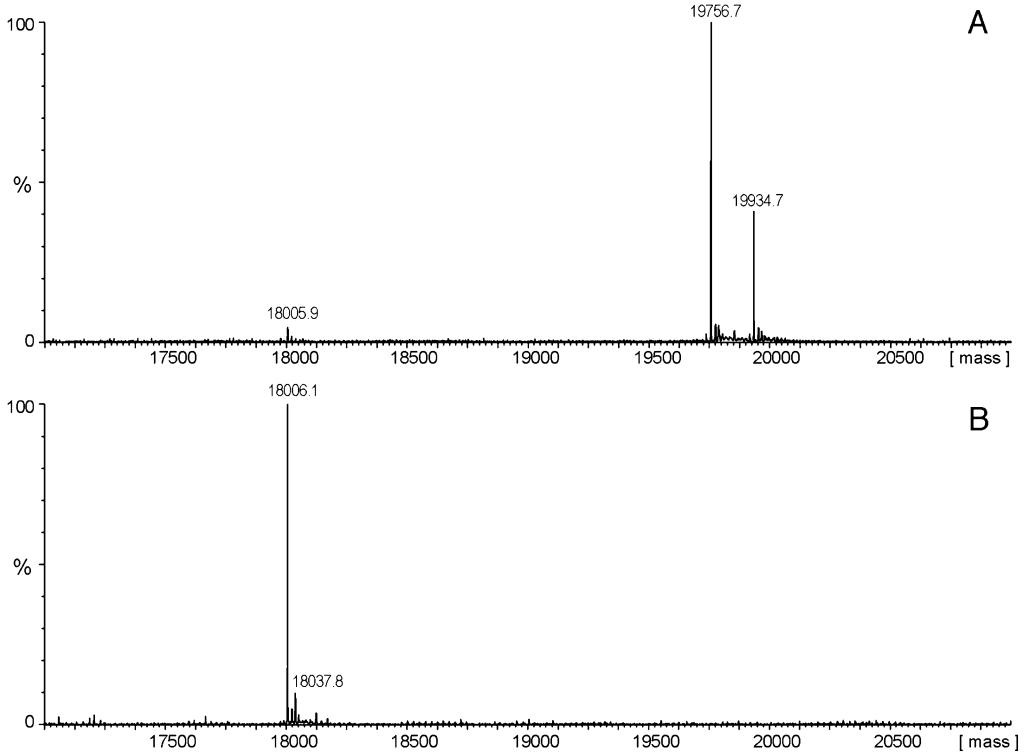

FIGURE 2: Deconvoluted (MaxEnt 1; Micromass, Manchester, U.K.) ESI mass spectrum of (A) His_6-tagged and (B) wild-type MOCS3-RLD protein. The His_6-tagged protein shows the presence of another species with a molecular mass increased by +178 Da.

In the mass spectrum a second minor species of the protein with an extra mass of 178 Da was determined (Figure 2A). Using MS/MS, an additional spectrum of the 21-fold charged molecular ion (Figure 3A) of the minor species was recorded and compared to the data from the main component (Figure 3B,C). While the C-terminal fragment ions were identical, all detected b-type fragments (starting from b_8) showed a mass increase of 178 Da. The most intense signals were found for the 4-fold charged $b_{26}-b_{29}$ ions assigned in Figure 3. These results clearly indicate a modification at the amino-terminal part of the protein. After removal of the His_6 tag by thrombin, a single mass of 18006.1 Da was detected exclusively, corresponding to the calculated mass of 18008.7 Da of the unmodified protein (with a difference of 2.6 Da) (Figure 2B). After tryptic digestion of MOCS3-RLD and ESI-MS/(MS) analysis, a mass increment of 178.0 Da was determined on the first glycine residue of the N-terminal peptide comprising amino acids $_2$GSSHHHHHHSSGLVPR$_{17}$. A report by Geoghegan et al. (*19*) showed the presence of a gluconoylic acid residue bound to the N-terminal GSS sequence preceding the six histidine residues of the His_6 tag introduced by the *E. coli* expression vector pET15b using NMR techniques for structural assignments. Since analysis of thiosulfate:sulfurtransferase activity of gluconoylated MOCS3-RLD compared to the form without the His_6 tag showed the same activity for all protein variants (data not shown), all further analyses were performed without cleavage of the His_6 tag.

Detection of Free Thiol Groups in MOCS3-RLD and Variants. To determine the accessibility of free SH groups in wild-type MOCS3-RLD compared to cysteine to alanine variants, site-directed mutagenesis was performed (Materials and Methods). Three protein variants were tested, one with the simultaneous replacement of C316/C324 to alanine, one replacing residues C316/C324/C365 by alanine, and finally a mutant exclusively replacing C412 by alanine. All proteins were incubated with DTNB, and the amount of formed 5-mercapto-2-nitrobenzoic acid was quantified. As shown in Table 1, under native conditions persulfurated MOCS3-RLD contains 1.56 accessible thiol groups in comparison to 1.88 thiol groups determined after incubation with cyanide. The number in thiosulfate-treated MOCS3-RLD in its native state might be lower as persulfide groups are relatively resistant toward DTNB reactivity (*20*). This difference is not present in the denatured protein variants, since the persulfide group is not stable to guanidinium chloride treatment. The same difference between the thiosulfate and cyanide-treated forms is seen for all protein variants with the exception of the active site C412A variant. Detection of free thiol groups under native and denaturing conditions showed a maximum of two per MOCS3-RLD (Table 1), indicating the formation of a disulfide bridge within the protein. To identify the cysteine residues involved in formation of the disulfide bond, the reactivity of MOCS3-RLD variants C316/C324 and C316A/C324A/C365A was compared toward DTNB. The results in Table 1 reveal that the disulfide bridge is formed between C316 and C324 in MOCS3-RLD.

To verify this, ESI-MS spectra of MOCS3-RLD wild type and the cysteine to alanine variants were recorded (Figure 4), and the masses were compared to the calculated masses (Table 2). While the masses of MOCS3-RLD wild type and variant C412A were lower by −2.7/−2.8 Da, the masses of variants C316A/C324A and C316A/C324A/C365A corresponded well to the calculated masses, which confirmed the results obtained by DTNB derivatization described above. Furthermore, the spectrum of the iodoacetamide-treated wild-type MOCS3-RLD protein (Figure 4B) revealed the dominance of the monocarboxamidomethylated derivative, con-

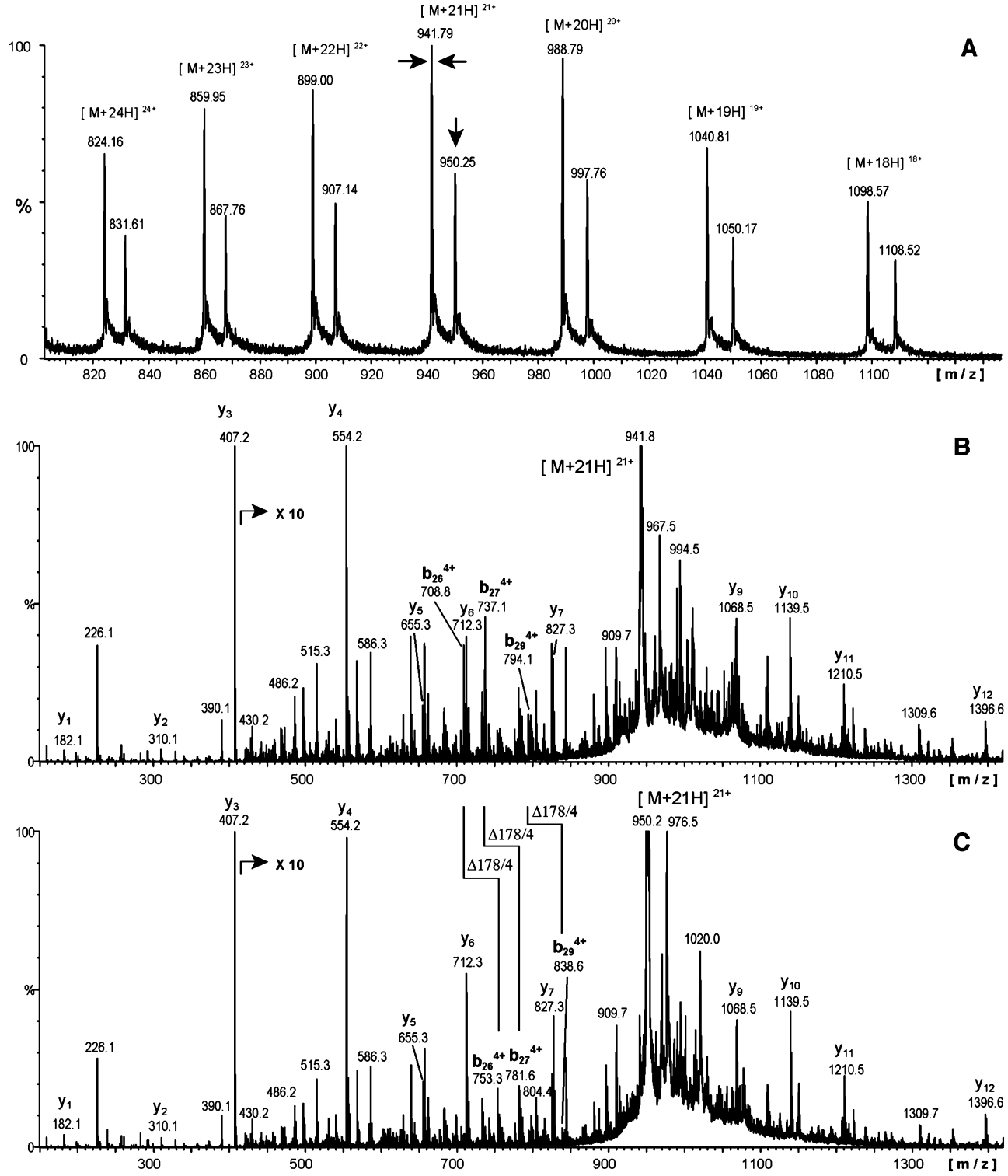

FIGURE 3: ESI mass spectra of the His_6-tagged wild-type MOCS3-RLD protein. (A) Double series of multiply charged molecular ions corresponding to the molecular masses depicted in Figure 2A. The precursor ions selected for MS/MS experiments are marked by arrows. Daughter ion spectra of the 21-fold charged molecular ion of the major (B) and minor protein form (C). From the carboxy-terminal fragment ions (y_n), which are identical for both species indicating an unmodified C-terminal part of both proteins, the expected amino acid sequence can be readily verified. The weak, but clearly detectable, amino-terminal sequence-specific fragments (b_n) exhibit a characteristic mass difference of 178 Da (depicted for the 4-fold charged $b_{26}-b_{29}$ ions, but being detectable with b_8), indicating a modification by an entity with a molecular mass of 178 at the amino-terminal part of the minor protein. Furthermore, the detected b-type fragments of the major protein form were 131 Da lower than calculated, indicating the loss of the amino-terminal methionine of the His_6 tag.

firming the presence of a disulfide bridge and suggesting a reduced accessibility of one of the remaining free cysteine residues. By ESI-MS analysis of trypsin-digested wild-type MOCS3-RLD, whose free cysteine residues were completely derivatized by reaction with iodoacetamide prior to digestion, the tryptic peptide containing C316 and C324 ($_{299}$GSHMRPTVTDLLDYEAF**C**GSSATDK**C**R$_{325}$) was readily detected at a mass of 2 Da lower than calculated for the underivatized peptide. Only trace amounts of the doubly carboxamidomethylated peptide variant were present, indi-

Table 1: Reactivity of DTNB with Sulfhydryl Groups of Folded and Unfolded MOCS3-RLD Variants

MOCS3-RLD variant	total no. of SH groups in protein	no. of SH groups detected with DTNB[a]			
		folded		unfolded[d]	
		+thiosulfate[b]	+cyanide[c]	+thiosulfate	+cyanide
wild type	4	1.56	1.88	2.02	2.11
C316A/C324A	2	1.34	1.99	2.11	1.92
C316A/C324A/C365A	1	0.36	0.89	1.04	0.87
C412A	3	0.92	1.07	0.89	0.93

[a] Total amount of SH groups determined by DTNB. The values were calculated by reading OD_{412} using a standard curve for cysteine. [b] Total amount of SH groups detected by DTNB after treating the protein with thiosulfate. Excess thiosulfate was removed from the mixture by gel filtration before the addition of DTNB. [c] Total amount of SH groups detected by DTNB after incubation of the protein with cyanide. Excess cyanide was removed from the mixture by gel filtration before the addition of DTNB. [d] Proteins were denatured with 5 M guanidinium chloride.

cating the predominant presence of a disulfide bridge between these cysteine residues. MS/MS (data not shown) of the putative disulfide-bridged peptide showed, as expected, no sequence-specific fragment ions from the bridged peptide part, unequivocally confirming our assignment. The peptide containing C365 ($_{344}$LLDSGAFHLLLDVRPQVEVDI**C**R$_{366}$) was detected exclusively in its carboxamidomethylated form, indicating a lack of participation in disulfide bridges, and the peptide containing C412 is comprehensively discussed later.

In Vitro Sulfurtransferase Activity of MOCS3-RLD Variants. With the finding that a disulfide bridge is formed between C316 and C324 in MOCS3-RLD, it was of interest to determine whether the formation of the disulfide is important for catalysis. In a previous study single cysteine to alanine variants in MOCS3-RLD were tested for their activity to provide the sulfur from thiosulfate for MPT biosynthesis (*12*). However, with single amino acid exchanges it remains possible that a disulfide bridge is formed between the remaining cysteine residues. Simultaneous exchange of all cysteine residues other than the active site C412 essential for catalysis should clearly show the involvement of other cysteine residues in the reaction. Table 2 shows the activity of MOCS3-RLD compared to the mutated variants in providing the sulfur for MPT production in vitro. In a fully defined in vitro system containing precursor Z, MOCS2A-OH, MoaE, MoeB, Mg-ATP, and thiosulfate-treated MOCS3-RLD wild type, C316A/C324A, or C312A/C324A/C365A variants, the same amount of MPT was detected in all incubation mixtures (Table 2). As shown previously, variant C412A exhibited no sulfurtransferase activity (*12*). This indicates that the formation of a disulfide bridge in MOCS3-RLD is not essential for the sulfur transfer to MOCS2A in vitro.

Direct Evidence of a Persulfide Group Formed on C412. If a persulfide group is formed on MOCS3-RLD during catalysis, a mass increase of 32 Da should be visible by ESI-MS after treatment of MOCS3-RLD with thiosulfate. In Figure 4 the deconvoluted ESI-MS spectra of different MOCS3-RLD variants after incubation with thiosulfate are shown. While all protein variants showed two forms of the protein with a mass difference of 32 Da after incubation with thiosulfate (and the corresponding gluconoylated derivatives; see above), only the C412A variant remained unchanged.

To directly show the transfer of a sulfur atom from thiosulfate to C412, the tryptic peptides of thiosulfate-treated MOCS3-RLD were mapped by ESI-MS. Surprisingly, mass shifts of 16, 32, 48, and 64 Da were observed on the target peptide $_{398}$QGTQEGAAVPIYVI**C**K$_{413}$. While the mass shifts of 16 and 32 can be explained by oxidation of a single sulfur atom, the compound with a mass shift of 48 and 64 Da must contain at least one additional sulfur atom. To prevent oxidation during sample digestion, the thiosulfate-loaded MOCS3-RLD was alkylated with iodoacetamide. The carboxamidomethylated peptide and a derivative with a mass increase of 32 Da were detected in a ratio of 1:1 without indications of oxidation reactions. Additionally, however, considerable amounts of the noncarboxamidomethylated free and persulfide-loaded species and their oxidized derivatives as described above were detected, confirming our finding by ESI-MS of the intact protein (Figure 4B), its tryptic C-containing peptides, and DTNB titration (Table 1) that C412 is less accessible than C365. Analysis of the fragment ions of the MS/MS spectra of both carboxamidomethylated peptide forms confirmed the expected amino acid sequence and clearly showed a modification at the cysteine residue of the heavier peptide (Figure 5). The oxidation of a single cysteine sulfur by two oxygen atoms could be excluded, taking advantage of the high mass accuracy of the MS instrument used. Since the mass difference between one sulfur and two oxygens is relatively large (0.018 Da), we used the clearly defined N-terminal fragment ions (b-series) of our target peptide not including the modified cysteine residue for mass calibration (lock masses) and obtained highly accurate masses of the carboxy-terminal fragments (y-series) including the modified cysteine residue and could in this way unequivocally demonstrate the presence of a persulfide group on C412.

DISCUSSION

In previous work the sulfurated form of MOCS3-RLD was shown to be able to transfer the sulfur from thiosulfate to MOCS2A in a defined in vitro system containing MOCS2A, MOCS2B, MoeB, and Mg-ATP (*12*). This observation provided the first evidence for a physiological substrate of a rhodanese-like protein in humans (*12*). Since the N-terminal MoeB domain of MOCS3 was found to be inactive for MPT biosynthesis, the activity of a separately purified His_6-tagged variant of MOCS3-RLD was characterized in detail by mass spectrometry in this study.

It was shown that during heterologous expression in *E. coli* as an His_6-tagged variant, in addition to cleavage of the N-terminal methionine, gluconoylation of the resulting N-terminal glycine residue introduced by the His_6-tag peptide from vector pET15b (Materials and Methods) was identified in significant amounts (10−50%). The modification of the

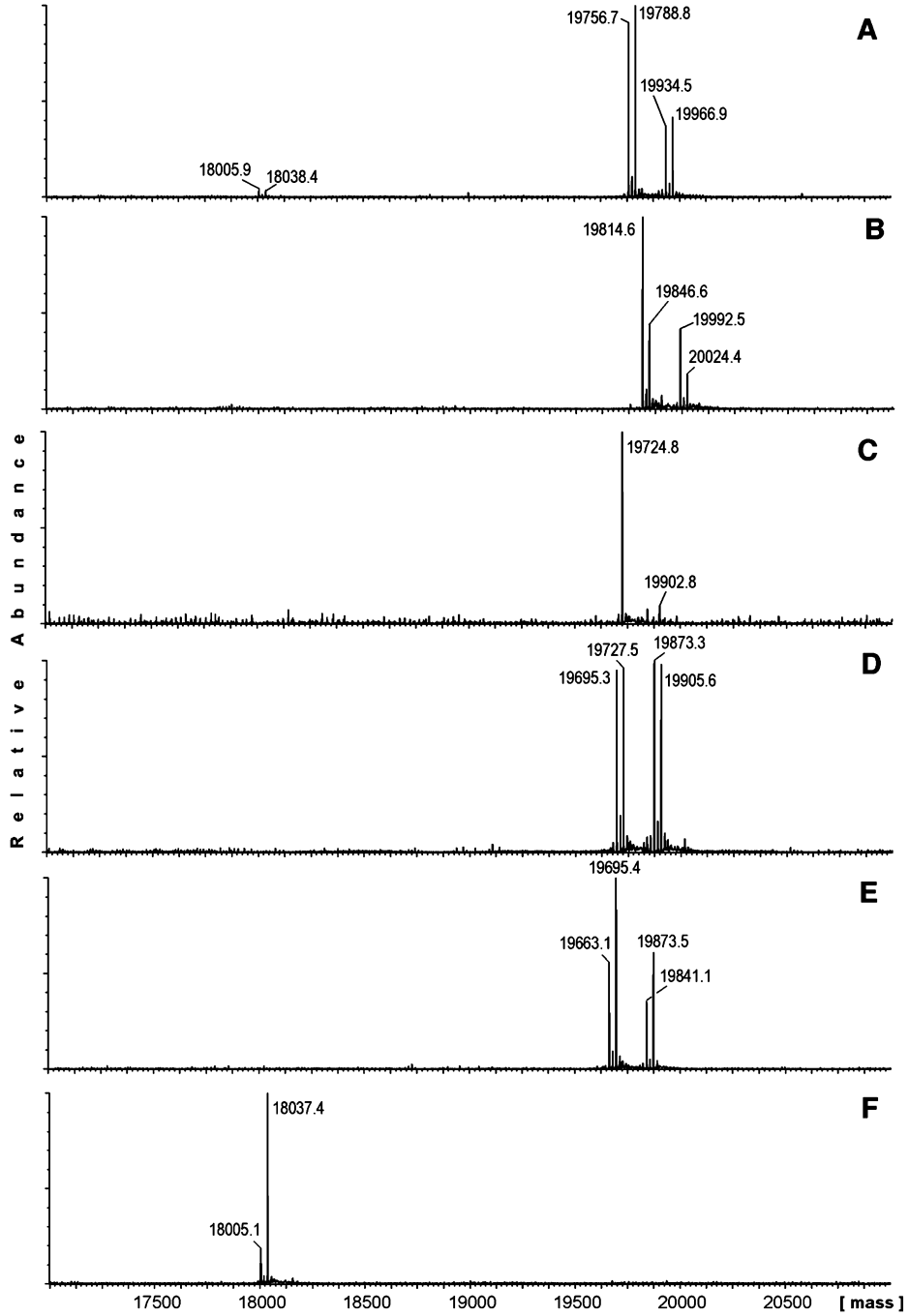

FIGURE 4: Deconvoluted (MaxEnt 1; Micromass, Manchester, U.K.) ESI mass spectra of partially persulfide loaded MOCS3-RLD variants (in parentheses their calculated average masses without disulfide bridges): (A) wild-type MOCS3-RLD + His_6 tag (19759.5), (B) monocarboxamidomethylated wild-type MOCS3-RLD (19816.6), (C) MOCS3-RLD-C412A + His_6 tag (19727.5), (D) MOCS3-RLD-C316A/C324A + His_6 tag (19695.4), (E) MOCS3-RLD-C316A/C324A/C365A + His_6 tag (19663.3), and (F) wild-type MOCS3-RLD (18008.7). The transfer of a sulfur atom is directly observable from the formation of a second protein molecular ion with a molecular mass of +32 Da with the exception of variant C412A (C). Apparently this protein lacks any sulfide binding capability. The reactivity of the gluconoylated species is identical to those of the unmodified protein in all cases. From the detected masses, which are slightly less than calculated for all forms with the exception of the C316A/C224A variant, the presence of a disulfide bridge between C316 and C324 is suggested (compare also Table 2).

protein had no negative effect on protein activity; however, the finding of a partially gluconoylated N-terminus of the purified protein is of general interest for further applications, since it might introduce heterogeneities into the protein (*21*).

The RL domain of MOCS3 contains four cysteine residues at positions 316, 324, 365, and 412 (Figure 1). Only residue C412 is conserved in homologous proteins from other organisms, and it is the expected active site cysteine residue of the conserved six amino acid loop characteristic for rhodanese-like proteins. Site-directed mutagenesis showed that exchange of C412 to alanine resulted in loss of sulfur transfer activity of the protein in vitro and in vivo (*12*); however, direct proof of the formed persulfide on C412 needed to be identified.

In this report we clearly showed by ESI-MS/MS analysis that a persulfide is formed on C412 during catalysis. After tryptic digestion of the protein the persulfide group was located on peptide $_{398}$QGTQEGAAVPIYVI**C**K$_{413}$. To avoid

Table 2: Calculated and Measured Masses and Determination of Activity of MOCS3-RLD and Variants

MOCS3-RLD variant	calculated mass (Da)[a]	measured mass (Da)[b]	mass difference (Da)	thiosulfate:cyanide sulfurtransferase activity (%)[c]	MPT formation (%)[d]
wild type	19759.5	19756.7	−2.8	100	100
C316A/C324A	19695.4	19695.3	−0.1	96.4 ± 5.4	106.3± 7.2
C316A/C324A/C365A	19663.3	19663.1	−0.2	71.2 ± 7.1	97.4 ± 8.5
C412A	19727.5	19724.8	−2.7	nd	nd

[a] Monoisotopic mass was calculated with ProtPram (http://us.expasy.org/tools/protparam.html). [b] ESI-MS measurements of protein variants pretreated with cyanide. [c] Thiocyanate (complexed with iron) was quantitated at A_{460nm} (*16*). Thiosulfate:cyanide sulfurtransferase activity of wild-type MOCS3-RLD was set to 100%. [d] MPT was quantitated as form A (*18*). The amount of MPT formed by wild-type MOCS3-RLD was set to 100%.

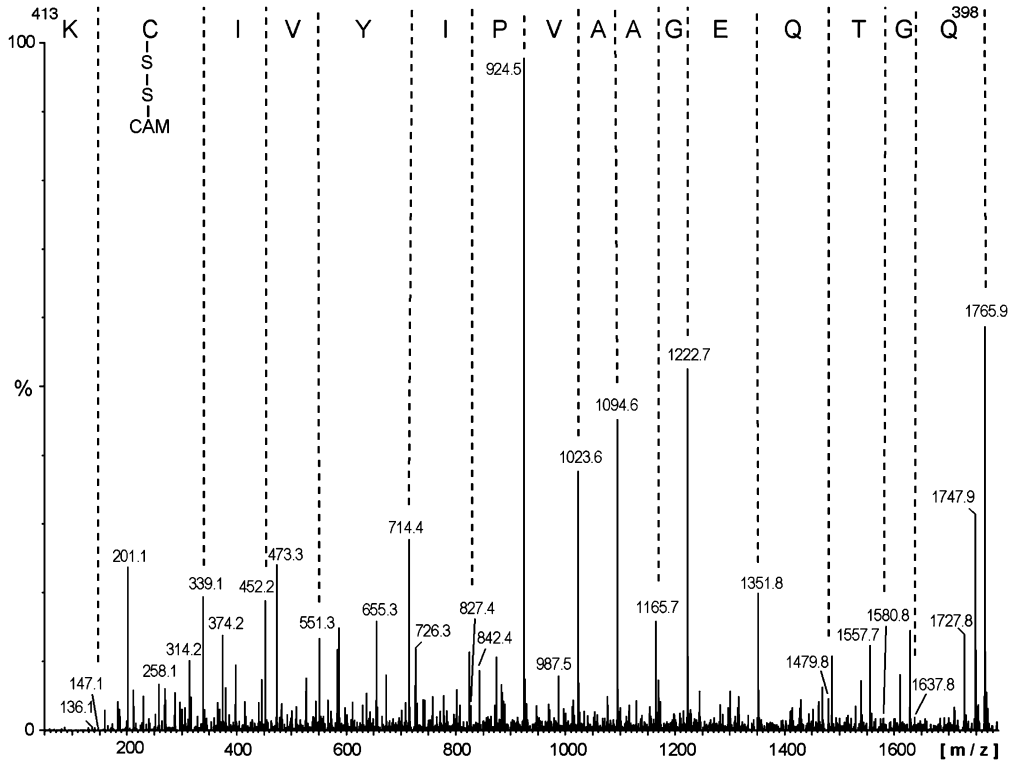

FIGURE 5: Computer-enhanced (MaxEnt 3; Micromass, Manchester, U.K.) daughter ion spectrum of the carboxamidomethylated tryptic peptide incorporating C412 showing the carboxy-terminal fragment ion series of the y_n type. From the masses of these fragment ions, the expected amino acid sequence could be deduced, and a modification by a carboxamidomethylated persulfide unit at the cysteine residue is unequivocally shown.

the observed oxidation of the persulfide group during sample preparation, persulfurated MOCS3-RLD was alkylated with iodoacetamide. The carboxamidomethylated peptide clearly showed a mass increase of 32 Da compared to the corresponding peptide obtained from a protein not treated with thiosulfate. These data unequivocally demonstrated the presence of a persulfide group on C412 in MOCS3-RLD by a method which has not been used before for this class of enzymes. No persulfide group was formed on any of the remaining three cysteines during catalysis. In addition, simultaneous mutagenesis of all cysteine residues except C412 showed that none of these are involved in the sulfur transfer reaction in vitro.

Detection of free thiol groups and additional mass spectrometry data demonstrated a disulfide bridge formed between C316 and C324 in MOCS3-RLD. These two cysteine residues are located in the linker region between the N-terminal MoeB-like domain and the C-terminal RL domain of MOCS3. Analysis of sulfurtransferase activity of a C316A/C324A variant showed that the disulfide bond is not essential for the sulfur transfer mechanism in vitro. It remains speculative if the disulfide bridge is also formed in vivo; however, it can be discussed that the two cysteines are involved in the mechanism of sulfur transfer to form the thiocarboxylate group on MOCS2A by getting the two domains in closer proximity.

In the case of bovine rhodanese, the three extra cysteine residues in addition to the active site cysteine C247 are thought to play important roles for different structural and functional aspects of the protein. Mutational analysis of these residues suggested an important role in protein folding, an influence on protein stability, and in the reactivation after oxidative inactivation (*6*, *22*). The possible formation of a disulfide bridge between two cysteine residues in proximity to C247 has been discussed (*23*) and appears to be a common feature in rhodanese-like proteins.

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