Functionality of Alternative Splice Forms of the First Enzymes Involved in Human Molybdenum Cofactor Biosynthesis*

Received for publication, January 29, 2002, and in revised form, March 6, 2002
Published, JBC Papers in Press, March 12, 2002, DOI 10.1074/jbc.M200947200

Petra Hänzelmann, Günter Schwarz, and Ralf R. Mendel‡
From the Institute of Plant Biology, Technical University of Braunschweig, D-38023 Braunschweig, Germany

In humans, genetic deficiencies of enzymes involved in molybdenum cofactor biosynthesis trigger an autosomal recessive and usually fatal disease with severe neurological symptoms. In each of the three biosynthesis steps, at least two proteins or domains are linked for catalysis. For steps 1 and 2, bicistronic mocs (molybdenum cofactor synthesis) mRNAs were found (mocs1 and mocs2) that have been proposed to encode two separate proteins (A and B). In both cases, the A proteins share a highly conserved ubiquitin-like double glycine motif, which is functionally important at least for the small subunit of molybdopterin (MPT) synthase (MOCS2A). Besides the bicistronic form of mocs1, two alternative splice transcripts were found, resulting in the expression of multidomain proteins embodying both MOCS1A, but without the double glycine motif, and the entire MOCS1B. Here we describe the first functional characterization of the human proteins MOCS1A and MOCS1B as well as the MOCS1A-MOCS1B fusion proteins that catalyze the formation of precursor Z, a 6-alkyl pterin with a cyclic phosphate, the immediate precursor of MPT in molybdenum cofactor biosynthesis. High level expression of MOCS1A and MOCS1B in Escherichia coli resulted in the formation and accumulation of precursor Z that was subsequently converted to MPT. We showed that for catalytic activity MOCS1A needs an accessible C-terminal double glycine motif. In the MOCS1A-MOCS1B fusion proteins lacking the MOCS1A double glycines, only MOCS1B activity could be detected. No evidence was found for an expression of MOCS1B from the bicistronic mocs1A-mocs1B splice type I cDNA, indicating that MOCS1B is only expressed as a fusion to an inactive MOCS1A. Comparative mutational studies of MOCS1A and the small subunit of the E. coli MPT synthase (MoaD) indicate a different function of the double glycine motifs in both proteins.

With the exception of nitrogenase, in all molybdenum enzymes studied so far, the molybdenum cofactor (Moco) consists of a mononuclear molybdenum coordinated by the diithiolen moiety of one or two of a family of tricyclic pyranopterinoid, the simplest of which is commonly referred to as molybdopterin (MPT) (1, 2). Moco biosynthesis is an ancient, ubiquitous, and highly conserved pathway leading to the biological activation of molybdenum. In humans, defects in Moco biosynthesis lead to the pleiotropic loss of the molybdenum enzymes sulfite oxidase, aldehyde oxidase, and xanthine dehydrogenase (3). Affected patients show neurological abnormalities such as attenuated brain growth, untreated seizures, and often dislocated ocular lenses, and they usually die shortly after birth (4). Moco biosynthesis in humans can be divided into three major steps (3). In step 1, mocs1 (molybdenum cofactor synthesis-step 1) has been reported to produce two enzymes (MOCS1A and MOCS1B) within a bicistronic transcript with two consecutive ORFs (5, 6). The mocs1 RNA structure suggests a translation reinitiation for the second mocs1B ORF. These two enzymatic activities catalyze the synthesis of precursor Z, an oxygen-sensitive 6-alkyl pterin with a cyclic phosphate, from a guanosine derivative, most likely GTP (7, 8) (Fig. 1A). In step 2, the conversion of precursor Z into MPT is catalyzed by MOCS3 and MPT synthase (mocs2) that encodes by leaky scanning the small (MOCS2A) and large (MOCS2B) subunits of this heteromeric enzyme via a single transcript with two overlapping ORFs (9). In the last step, molybdenum is incorporated into MPT by the two-domain protein gephyrin (10, 11). mocs1 and mocs2 are defective in patients with complementation group A and B, respectively (12). In addition, a third type of Moco deficiency, type C, is caused by mutations in the gephyrin gene (13).

Besides the bicistronic form of mocs1 mRNA (splice type I) (3, 5, 6) monocistronic transcripts were found (14) that are spliced in a way that bypasses the normal termination codon of mocs1A, resulting in a single multidomain protein embodying both MOCS1A and MOCS1B (splice types II and III) (Fig. 1B). The latter are created by a variety of splicing mechanisms like alternative splice donors, alternative splice acceptors, and exon skipping. This coexpression profile was observed in many vertebrates and invertebrates (14).

In all organisms studied so far, two proteins catalyze the synthesis of precursor Z (6, 15–17). The plant and human orthologs of the Escherichia coli proteins MoaA (Cnx2, MOCS1A) and MoaC (Cnx3, MOCS1B) show N-terminal extensions of yet unknown function. MOCS1A and homologous proteins are characterized by two cysteine clusters probably involved in iron-sulfur (FeS) cluster binding (18–20). Based on sequence similarities to proteins like bietin synthase, pyruvate formate lyase-activating enzyme, anaerobic ribonucleotide reductase-activating enzyme, and lysine 2,3-amimonutase, MOCS1A belongs to a new superfamily called "radical S-adenosylmethionine (SAM)" proteins (21). It is proposed that they generate a radical species by reductive cleavage of SAM through an unusual FeS cluster. Proteins belonging to this
family catalyze diverse reactions, including unusual methylations, isomerization, sulfur insertion, ring formation, anaerobic oxidation, and protein radical formation (21). In addition, MOCS1A and homologous proteins from eubacteria and eu-karyotes are characterized by a highly conserved C-terminal double glycine motif that is absent in the alternatively spliced MOCS1A-MOCS1B fusion proteins. A similar motif is found in the small subunits of MPT synthases that are C-terminally thiocarboxylated in order to catalyze the sulfur transfer to precursor Z, resulting in the formation of the MPT dithiolene (22, 23). The bacterial MOCS1B homolog MoaC is a hexamer composed of three dimers with a putative active site located at the dimer interface (24).

It has been proposed that the formation of precursor Z occurs by an alternative cyclohydrolase-like reaction (7, 8). As in the pathways of folate, riboflavin, and biotinynthesis, a guanosine derivative serves as the initial biosynthetic precursor (25, 26). During precursor Z synthesis, all carbon atoms of the guanosine are utilized, because the imidazole ring carbon 8 is retained and incorporated in a rearrangement reaction as the first carbon of the precursor Z side chain (7, 8). This is different from all other pathways and indicates a novel route for pterin synthesis.

Here we describe the first functional characterization of the human proteins MOCS1A and MOCS1B as well as the MOCS1A-MOCS1B fusion proteins derived by alternative splicing. Despite their N-terminal extensions, MOCS1A and MOCS1B were able to restore Moco biosynthesis in E. coli moaA− and moaC− mutants, respectively, which enabled us to study the human proteins by site-directed mutagenesis. Functional analysis was performed by nitrate reductase reconstitution as well as by monitoring the cellular levels of precursor Z and MPT. High level expression of MOCS1A and MOCS1B in E. coli moaA− and moaC− mutants resulted in the formation and accumulation of precursor Z that was subsequently converted to MPT. We showed that for catalytic activity MOCS1A needs an accessible C-terminal double glycine motif. In the MOCS1A-MOCS1B fusion proteins lacking the MOCS1A double glycine motif, only MOCS1B activity could be detected. No evidence was found for an expression of MOCS1B from the bicistronic mocs1A-mocs1B splice type I cDNA, indicating that MOCS1B is only expressed as a fusion to an inactive MOCS1A.

Comparative mutational studies of MOCS1A and the small subunit of the E. coli MPT synthase (MoaD) indicate a different function of the double glycine motifs in both proteins.

EXPERIMENTAL PROCEDURES

Materials, Plasmids, and Bacterial Strains—Oligonucleotides for PCR and sequencing were purchased from Invitrogen. Restriction enzymes and T4 DNA ligase were purchased from Promega (Madison, WI), and P沃 polymerase was from Peflab (Erlangen, Germany). DNA sequencing was carried out with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 310 cycle sequencer (PerkinElmer Life Sciences) with a pop 6 polymer. The T3 RNA polymerase-based bacterial pQE80 expression vector was purchased from Qiagen (Hilden, Germany), and the mammalian expression vectors pcDNA3 and pEGFF-C1 were from Invitrogen (Groningen, The Netherlands) and CLONTECH (Heidelberg, Germany), respectively. The E. coli wild type strain MC4100 (araD139 argF-lacU169 rpsL150 relA1 flbB3501 deoC1 ptsF25 rbsR) (27) as well as the moaA−, moaC−, and moaD− KB mutant strains [F− thr, leu his pro arg thi ade gal lacY malE xyl ara mtl str T′, A′] were kindly provided by David Boxer (Department of Biochemistry, Medical Science Institute, Dundee University, UK). The moaD− strain MJ431 (M7 (F− rpsL) chlM) (28) was kindly provided by Gérard Giordano (Centre National de la Recherche Scientifique, Marseille, France). The plasmids pJR11 (E. coli moaABCDE in pGEM-T easy) were kindly donated by Jochen Reiss (Institute of Medical Physics and Biophysics, University of Münster, Germany). Polyclonal antibodies raised against recombinant MOCS1A and MOCS1B were generated by BioScience (Göttingen, Germany).

Construction of Expression Plasmids—mocs1A and mocs1B were cloned by PCR from pJRMOCS1 and moaD (29) from pJR11, respectively. The published gene sequences (GenBankTM AJ224328 (mocs1) and X70420 (mocsABCDE)) (5, 16) were used to design oligonucleotides that permitted cloning into the BamHI and SalI sites of the multiple cloning region of the pQE80 expression vector. The resulting plasmids were designated pPH80MOCS1A, pPH80MOCS1B, and pPH80MoaD. mocs1 splice types II and III were created by a two-step PCR procedure from pJRMOCS1 and cloned into the BamHI and SalI sites of the multiple cloning region of the pQE80 expression vector. The published gene sequences (GenBankTM AF214022 (mocs1 type II) and AF214023 (mocs1 type III)) (14) were used to design oligonucleotides that permitted construction of the different splice types. First, two separate PCRs were conducted with the mocs1A-BamHI sense primer and the antisense primers 5′-GATGAGGATCGGGCCGG-3′ (mocs1A-II) or 5′-TG- CATGGTGCCCCGCTTCTTT-3′ (mocs1A-III), respectively, and with the sense primers 5′-AAGAACCGGCCCATGATCCTCCAAATCCG-3′ (mocs1B-II) or 5′-GGAGCAGAAGAGCGGACCGATCGAAGATTTTGGATGTTCCCAATCCCG-3′ (mocs1B-III), respectively, and the mocs1B-SalI antisense primer. Both products of each splice type were used as the template for the second round of PCR, which was carried out with the mocs1A-BamHI sense primer and...
the mocs1-Sal1 antisense primer. The resulting plasmids were designated pPH80MOCS1III and pPH80MOCS1III.

Site-directed Mutagenesis of the C-terminal Double Glycine Motifs of MOCS1A and Moa—Oligonucleotides were designed for cloning into the BamHI and SalI sites of pQE80 as depicted under “construction of expression plasmids.” Generation of single and double amino acid substitutions or insertion and deletion of amino acids were achieved by modifying the SalI antisense cloning primer. The following mutations were created: (a) MOCS1A: G384A, G384S, G384C, G384V, G384D, G385A, G385P, G385C, delG385, delIGG, GGG*, GGA*, GGV*, AA*, and A*; (b) MoaD: G80A, G80V, G81A, delG81, and GGG*. For the exchange of the mocs1A nonsense codon to an alanine residue, resulting in a MOCS1A fusion protein, a two-step PCR procedure with pBR-MOCS1 as the template was carried out as described under “construction of expression plasmids.” The identity of all mutations was confirmed by DNA sequencing.

Protein Expression and Immunoblot Analysis—All expressions were conducted in LB medium at 30 °C. Cultures were induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when cells had reached an optical density of 0.6. After 4 h of growth, cells were harvested and resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol) and sonicated. After centrifugation, the soluble and insoluble fractions were separated by SDS-PAGE using a 12% polyacrylamide gel. Immunoblotting on polyvinylidene difluoride membranes was carried out with primary polyclonal MOCS1A (1:250 diluted serum) and Cnx3 (17) using a fluorescence detector (excitation at 370 nm, emission at 450 nm, gain 17). The membranes were probed with horseradish peroxidase-conjugated secondary antibody, and bands were visualized by the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium detection system (Promega).

Complementation of E. coli mocaA, mocaC, and mocaD—Mutants—For functional complementation of Moco mutants, E. coli KB strains were transformed with the corresponding expression plasmids. For quantitative determination of nitrate reductase activity, E. coli KB strains were grown anaerobically at 37 °C in 50 ml of LB medium containing 0.4% (w/v) nitrate. Where indicated, protein expression was induced with 0.1 mM IPTG. Nitrate reductase activity in crude cell extracts was determined by a spectroscopic assay using benzyl viologen as described (30). Protein concentrations were determined using the Lowry technique (31).

Analysis of Precursor Z and MPT in Crude Cell Extracts—MPT and precursor Z were detected as dephospho form A and compound Z in crude cell extracts, respectively, according to the method described (28, 32). Oxidation, dephosphorylation, diethyl-2-hydroxypropylaminoethyl (QAE) Sephadex A-25 chromatography, and HPLC analysis of dephospho form A were performed as described (33). Oxidations were carried out with 0.3–0.5 mg of crude cell extract. Compound Z was isolated by the same protocol, but elution from the diethyl(2-hydroxypropyl)aminoethyl matrix was performed with 0.5 ml of the elution buffer containing 10 mM potassium phosphate, pH 3.0, as mobile phase using a C18 reversed phase column. The precursors of MPT in comparison with the wild type strain MC4100 (Fig. 2, A–D). The E. coli strain moaA strain (moaA−) to identify precursor Z in crude cell extracts (32). Overexpression (plus 0.1 mM IPTG) of MOCS1A in the mocaA− mutant strain resulted in a 5-fold accumulation of precursor Z in comparison with the MJ7mchiM strain and also in a 5-fold higher level of MPT in comparison with the wild type strain MC4100 (Fig. 2E). Under low expression conditions (without IPTG), no precursor Z but wild type levels of MPT could be detected that are the result of a basal expression of MOCS1A due to a leaky promoter. In contrast to MOCS1A, overexpression of MOCS1B resulted only in wild type levels of MPT and amounts of precursor Z comparable with the MJ7mchiM strain. These data show the complete functionality of MOCS1A and MOCS1B in catalyzing the formation of precursor Z that can subsequently be converted to MPT. Furthermore, the high level of precursor Z accumulation under the action of MOCS1A indicates that MOCS1A is the catalytically active and rate-limiting protein in precursor Z formation.

MOCS1A and homologous proteins are characterized by two highly conserved cysteine clusters, one in the N-terminal (consensus sequence: CX₃CX₄X₃(C) and one in the C-terminal (consensus sequence: CX₆CX₁₀X₂(C) region of the protein, both of which are proposed to be involved in FeS cluster binding (18, 19). The N-terminal cysteine cluster is the major feature of the radical SAM superfamily (21) harboring a [4Fe-4S] cluster, whereas the C-terminal cluster is unique for MOCS1A and homologous proteins and is absent in all other members of the radical SAM family. Purified MOCS1A was
brownish in color and contained 4 mol of iron/mol of protein, indicating the presence of a [4Fe-4S] cluster. 2

Characterization of Alternative mocs1 Splice Forms—It was recently shown that diverse splicing mechanisms fuse the evolutionarily conserved bicistronic mocs1A and mocs1B ORFs (14). Both identified alternative splice types II and III have deletions of 15 or 63 nucleotides in comparison with the bicistronic splice type I, leading to fusion proteins lacking the con-

FIG. 2. Functional reconstitution of the E. coli Moco mutants moaA− (KB2037) and moaC− (KB2066) with MOCS1A and MOCS1B: HPLC analysis of precursor Z and MPT in crude cell extracts. A and B, identification of precursor Z determined by measurement of its stable oxidized derivative, compound Z, in reversed phase HPLC. I, E. coli moaA− (KB2037) (A) and moaC− (KB2066) mutant (B); II, E. coli wild type strain MC4100; III, E. coli mutant strain MJ7chIM; IV, E. coli moaA− and moaC− cells expressing MOCS1A (A) and MOCS1B (B). C and D, identification of MPT determined by measurement of its stable oxidized derivative, dephospho form A, in reversed phase HPLC. I, E. coli moaA− (KB2037) (C) and moaC− (KB2066) mutant (D); II, E. coli wild type strain MC4100; IV, E. coli moaA− and moaC− cells expressing MOCS1A (C) and MOCS1B (D). Fluorescent material was detected by excitation at 370 nm, and emission was detected at 450 nm. E, levels of compound Z (white bars) and dephospho form A (black bars) of E. coli wild type (WT) strain MC4100, E. coli mutant strain MJ7chIM, E. coli moaA− and moaC− cells transformed with pQE80 vector (control), E. coli moaA− and moaC− cells expressing wild type MOCS1A and MOCS1B. Integrated peak areas were calculated per mg of protein. The amounts of compound Z and dephospho form A produced by MOCS1A were set to 100%. Assays were performed in triplicate, and S.D. values are shown by error bars. n.d., not detected.

2 P. Hänelmann, unpublished results.
served C-terminal double glycine motif of MOCS1A (Fig. 1). To investigate the functional properties of these fusion proteins on protein level, both alternative mocs1 splice types were reconstructed from splice type I by PCR based on the published nucleotide sequence (GenBankTM AF214022 and AF214023) and subcloned into the bacterial pQE80 expression vector. For functional characterization, MOCS1 splice type II and splice type III were expressed in the E. coli moaA− mutant KB2037 (plus 0.1 mM IPTG) or moaC− mutant KB2066 (plus 0.1 mM IPTG), and their activities were determined by measuring nitrate reductase activities in crude cell extracts (Fig. 3A). The MOCS1B domain of both fusion proteins was still functionally active (Fig. 3A). In contrast, the MOCS1A domain was not able to restore nitrate reductase activity in the moaA− mutant (Fig. 3A), indicating the importance of the C terminus including the double glycine motif. This finding is in agreement with a mutation of the first glycine to a serine within the double glycine motif leading to human Moco deficiency (3).

To prove whether or not MOCS1A activity can be restored by introduction of the missing C terminus, an artificial MOCS1A-MOCS1B fusion protein still containing both glycine residues was constructed by the exchange of the mocs1A nonsense codon to an alanine residue (1A-GGA-1B). However, the MOCS1A domain of this form was also inactive (Fig. 3A), showing that the C terminus of MOCS1A must be accessible for interaction or catalysis. This conclusion could be confirmed by a monocistronic MOCS1A variant containing an additional Val at the C terminus (GGV+) that was also completely inactive (Fig. 3A). These data demonstrate the importance of the bicistronic mocs1 transcript that leads to an unfused MOCS1A with a free accessible C terminus. Both fusion proteins (splice type II, splice type III) and the artificial fusion protein (1A-GGA-1B) with the double glycine motif were stably expressed in E. coli (Fig. 3B), and they were characterized by a brownish color, indicating a proper protein folding leading to the insertion of the FeS cluster despite the inactivity of the MOCS1A domain.

Expression of Alternative MOCS1 Splice Forms in HeLa Cells—Based on phylogenetic data combined with RNA blot analysis, there is evidence that the bicistronic form (splice type I) is likely to produce only MOCS1A and not MOCS1B (14). MOCS1B would then be translated exclusively as a fusion to MOCS1A (splice types II and III) (14). To investigate which ORF is translated from the bicistronic mocs1 cDNA, the mocs1 splice type I cDNA was cloned into the mammalian expression vector pcDNA3, transfected, and transiently expressed in HeLa cells. Immunoblot analysis showed expression of MOCS1A, but no MOCS1B could be detected (Fig. 4A). For transfection of splice types II and III, GFP fusion proteins were constructed, which enabled us to monitor efficient protein expression by fluorescence microscopy. Both fusion proteins were stably expressed and not degraded or proteolytically cleaved into separate MOCS1A and MOCS1B domains (Fig. 4B). Therefore, we conclude that MOCS1B is only expressed as a fusion to MOCS1A in mammalian cells.

Characterization of the C-terminal Double Glycine Motif of MOCS1A—We have shown that the C terminus of MOCS1A with the double glycine motif must be accessible for interaction or catalysis. A secondary structure prediction of MOCS1A and homologous proteins indicates that the C-terminal double glycine motif is located in a short solvent-accessible loop adjacent to a β-strand. So far, no catalytic or structural function for the C-terminal double glycine motif of MOCS1A is known. Therefore, we decided to examine the role of both glycines by determining the effect of deletion, extension, and a number of point mutations concerning both glycines as indicated in Fig. 5A. All generated MOCS1A variants showed a similar expression pattern (amount and contribution of soluble and insoluble protein) as the wild type protein, indicating no significant structural changes of MOCS1A that might lead to protein instability and/or altered solubility (Fig. 6A). For functional characterization, MOCS1A wild type or mutant forms were expressed (plus 0.1 mM IPTG) in the E. coli moaA− mutant KB2037, and their activities were determined by measuring nitrate reductase activity in crude cell extracts (Fig. 6A). Surprisingly, both terminal glycine residues could be exchanged separately to alanine (G384A and G385A), or one glycine could be removed (G+) without any loss of activity (Fig. 6A). Even an additional glycine at the C terminus (GGG+) showed no effect on nitrate reductase activity (Fig. 6A). However, the MOCS1A variant with an additional alanine at the C terminus (GGGA+) had only 19% activity (Fig. 6A). Deletion of both glycines (delGG) completely abolished activity, indicating again the importance of the C-terminal double glycine motif (Fig. 6A). Large amino acids at both positions decreased activity significantly (G384I, G384C) or led to inactivity (G384V, G384D, G385C, G385P), indicating that small amino acids like glycine or alanine must be present at the C terminus (Fig. 6A). However, at least one glycine is necessary, since a deletion of the last glycine combined with an exchange of the penultimate glycine to alanine

![Fig. 3. Functional reconstitution of the E. coli Moco mutants moaA− (KB2037) and moaC− (KB2066) with MOCS1A, MOCS1B, and alternative MOCS1 splice types and variants. A, nitrate reductase (NR) activity of E. coli wild type (WT) strain MC4100 (white bar), E. coli moaA− and moaC− cells transformed with pQE80 vector (control), E. coli moaA− and moaC− cells expressing MOCS1A and MOCS1B (black bar), and splice types and variants (gray bars). Assays were performed in triplicate, and S.D. values are shown by error bars. Expression was induced with 0.1 mM IPTG. +, stop; n.d., not detected. B, expression (plus 0.1 mM IPTG) of alternative splice types and variants in E. coli moaA− and moaC− mutants. The soluble (s) and insoluble (i) fractions of E. coli crude cell extracts were separated by SDS-PAGE and immunoblotted with polyclonal MOCS1A and MOCS1B antibodies.](http://www.jbc.org/content/full/18307/15/13756/F1.large.jpg)
Alternative mocs1 Splice Forms

**Fig. 4.** Transient expression of different mocs1 splice types in HeLa cells. HeLa cells were transfected with the different splice type constructs, and after transient expression crude cell extracts were separated by SDS-PAGE and immunoblotted with polyclonal MOCS1A and MOCS1B antibodies as indicated below the immunoblots. A, expression of MOCS1 splice type I. c, control (recombinant MOCS1A and MOCS1B (10 ng)); E, HeLa extract (50 μg); B, expression of MOCS1 splice type II (II) and III (III) fused to GFP.

(A*) resulted in a significant decrease of activity (50% of wild type), and a replacement of both by alanine (AA*) led to complete inactivity (Fig. 6A). Under conditions without induced expression (without IPTG), which perhaps represent the *in vivo* conditions in humans, only the MOCS1A variants G385A (GA*) and delG385 (G*) showed significant activities of 37 and 45% in comparison with wild type MOCS1A (GG*) (Fig. 7A). However, under those conditions the G384A (AG*) variant, which was fully active after induction with IPTG, had no activity, and the MOCS1A-GGG* variant showed activities of only 14% in comparison with wild type MOCS1A (Fig. 7A).

For further examination of this functional differences under low and high expression conditions, we determined the levels of precursor Z (measured as compound Z) and MPT (measured as dephospho form A) for the G384A, G385A, delG385, and GGG* variants (Fig. 6B). Under low expression conditions, the amounts of precursor Z and MPT were below the limit of detection. Under high expression conditions (Fig. 6B), the G385A, G*, and GGG* variants produced high levels of MPT; however, the precursor Z levels were already strongly decreased in comparison with wild type MOCS1A. In particular, the G385A variant (no activity under low expression conditions but full activity under high expression conditions) had only MPT levels and no detectable precursor Z. Both values are very similar to the wild type strain MC4100. However, these levels are already sufficient for complete activation of nitrate reductase under high expression conditions (Fig. 6A). Since in the *moaA* strain carrying wild type MOCS1A, the MPT level is 5-fold decreased at low level expression conditions as compared with high level expression (Fig. 2E), it is obvious that low level expression conditions led to the observed inactivity of the G384A variant and the strongly reduced activities of the other variants. In summary, these data show that despite 100% reconstituted nitrate reductase activities the level of MPT and especially of precursor Z is already reduced in the different double glycine variants. Based on these results, we conclude that the double glycine motif of MOCS1A is functionally essential, and some of our mutations seem to be dependent on the intracellular amount of protein because they can be rescued at least partially by higher expression levels.

Functional Comparison of MOCS1A with the Small Subunit of MPT Synthase—Interestingly, MOCS2A, Cnx7, and MoaD, the small subunits of human, plant, and bacterial MPT synthase, respectively, are proteins with a ubiquitin-like fold (36) that contain a C-terminal double glycine motif. The function of this motif in the reaction of precursor Z to MPT is well established for *E. coli* MoaD. In this case, the last glycine is adenylation by the action of MoeB (37, 38) and subsequently thio-carboxylated (22, 23). The synthesis of the dithiolene group of MPT is catalyzed by the heterotetrameric MPT synthase (MoaD-MoaE) that transfers the sulfur from the thio-carboxylate to precursor Z (23). The presence of a functionally essential C-terminal double glycine motif in MOCS1A as well as MOCS2A suggested similar functional properties of both proteins. Therefore, we decided to carry out comparative site-directed mutagenesis of the double glycine motif of the small subunit of MPT synthase (Fig. 5B). Since the human MOCS2A protein is not capable of restoring nitrate reductase activity of a *moaD* mutant, these experiments were done with the bacterial MoaD protein. For functional characterization, the *E. coli* moaD mutant KB2047 was reconstituted with wild type or mutant MoaD, and reconstitution was determined by measuring nitrate reductase activities. For comparison with MOCS1A, reconstitutions were done in the absence of IPTG (low expression) as well as under conditions with induced expression (plus 0.1 mM IPTG, high expression) (Fig. 7, A and B). At low expression levels (Fig. 7A), the exchange of the last glycine to alanine (GA*) as well as the addition of a third glycine to the C terminus (GGG*) led in both cases to the same significant decrease of activity in comparison with the wild type protein (GG*). Remarkable differences between both proteins occurred (i) when the penultimate glycine was changed to alanine (AG*) and (ii) after the deletion of the C-terminal glycine (G*) (Fig. 7A). Whereas the GG → AG exchange resulted in a total loss of MOCS1A activity, MoaD was almost fully active, indicating that in MOCS1A the more essential residue is the penultimate glycine. Similar results were obtained for the VG* mutant, which showed 30% wild type activity in MoaD. On the other hand, a deletion of the terminal glycine residue (G*) completely abolished MoaD activity because of the loss of thio-carboxylation and/or proper MPT synthase assembly, whereas MOCS1A showed significant activity (45% of wild type).

In contrast to MOCS1A, the MoaD variants did not show the observed effect of increased activities under conditions with induced expression as described above. Under these conditions, the MOCS1A variants AG*, GA*, G*, and GGG* gave nitrate reductase activities comparable with wild type MOCS1A, indicating a compensation of the particular mutations (Fig. 7B). The MoaD-G* and MoaD-GGG* variants were completely inactive, and the AG* and GA* variants were comparable with

---

G. Gutzke, personal communication.
low level expression conditions (Fig. 7B). In summary, our data indicate a different function of the C-terminal double glycine motifs in MOCS1A and MoaD.

**DISCUSSION**

Moco biosynthesis is an ancient, ubiquitous, and essential pathway in pro- and eukaryotes. In humans, at least two proteins and/or domains are genetically and biochemically linked in all three reaction steps (3). Each of the first two steps is catalyzed by two proteins that are encoded via bicistronic mRNAs (mocs1 and moocs2) (6, 9). In both cases, the proteins encoded by the 5'-ORF (MOCS1A and MOCS2A) share a highly conserved C-terminal double glycine motif. For mocs2 it was demonstrated that both ORFs (MOCS2A and MOCS2B) are translated in order to assemble into MPT synthase (9). For the *E. coli* homolog of MOCS2A (MoaD) it is known that it is first activated by C-terminal adenylation (37, 38) and subsequently sulfated via thiocarboxylation (22, 23). This conserved reaction mechanism was also found for bacterial ThiS in thiamin biosynthesis (39) and has served as the evolutionary basis for ubiquitin-dependent protein degradation (40). Also, MOCS1A needs an accessible C-terminal double glycine motif for activity (Fig. 6).

Our comparative mutational study of the C-terminal double glycine motifs of MOCS1A and MoaD showed two remarkable differences between both proteins under *in vivo* conditions (Fig. 7A). (i) In the case of MOCS1A, the penultimate glycine could not be substituted by any other amino acid, even by alanine, whereas an alanine mutation in MoaD was almost fully active. (ii) In contrast, the deletion of the last glycine led to inactivity of MoaD, whereas MOCS1A still showed 45% activity. Therefore, our data indicate a different function of the C-terminal double glycine motifs in MOCS1A and MoaD. MoaD clearly needs two glycine residues, and a deletion of the last glycine results in a shorter C-terminal loop that is probably no longer able to interact with MoeB and/or MoeE (36, 38). Because MoaD participates in strong protein-protein interactions where the highly conserved C terminus binds to either MoeB (for adenylation) or MoeE (for sulfur transfer) (36, 38), we conclude that the penultimate glycine residue fulfills a more structural function and the terminal glycine is directly involved in catalysis.

Furthermore, it was unexpected that despite the GG -> GA exchange MoaD showed a significant activity of about 35% in comparison with the wild type protein, indicating that adenylation/thiocarboxylation also can occur on an alanine residue, however with lower efficiency. In contrast, MOCS1A needs for catalytic activity mainly the penultimate glycine that plays only a minor role for MoaD activity. Even a shortening of the C terminus does not have the same dramatic effect as observed in MoaD. Therefore, it might be that in contrast to MoaD, the MOCS1A C-terminal glycine is not involved in catalysis.

Like biotin synthase and lipoate synthase, the putative radical SAM protein MOCS1A is participating in a pathway with sulfur transfer (21). However, despite the presence of a MOCS2A-like C-terminal double glycine motif, MOCS1A does not act directly on sulfur because precursor Z was identified as the sulfur-free precursor of MPT (41). In addition, no MoeB-like protein that could be involved in adenylation of MoeA (MOCS1A) is known (42). MoeB itself cannot be involved in this process, since moeB mutants accumulate precursor Z (43). Therefore, an adenylation/thiocarboxylation reaction that could facilitate the proposed rearrangement reaction of the GTP imidazole C-8 by generation of a transient formyl thioester (8) is unlikely. A more likely function of the double glycine motif in MOCS1A might be the interaction with MOCS1B, forming a stable MOCS1A-MOCS1B protein complex or a transient complex during catalysis as in the MoaD-MoaE and MoeB-MoaD protein complexes (36, 38). The C-terminal loop of MOCS1A might interact with the active site of MOCS1B, which is formed by two monomers in the homologous MocA protein from *E. coli* (24). However, to this end, we cannot exclude an involvement of the double glycine motif in a radical-based reaction mechanism catalyzed by the putative radical SAM protein MOCS1A. The functional importance of the C-terminal double glycine motif presented in this study for MOCS1A ap-
plies also to \textit{E. coli} \textit{MoaA} \textsuperscript{4,5} and is believed to be universal for all eubacteria and eukaryotes. It must be noted that the C-terminal double glycine motif essential for MOCS1A activity is a property that is not conserved in archaea, probably indicating a different catalytic mechanism in archaea.

Besides the bicistronic form of \textit{mocs1}, two alternative splice transcripts were found in which the termination codon of \textit{mocs1A} is bypassed, resulting in the expression of MOCS1A-MOCS1B multidomain proteins \textsuperscript{(14)}. When analyzing the functional properties of both splice types, it turned out that the MOCS1B domain remained active (Fig. 3A). Due to the lack of the double glycine motif, the MOCS1A domain was no longer able to catalyze the conversion of GTP to precursor Z (Fig. 3A).

Up to now, there was no \textit{in vivo} evidence supporting the independent translation of MOCS1A and MOCS1B polypeptides from the bicistronic \textit{mocs1} mRNA. Transient expression of the bicistronic form of \textit{mocs1} in HeLa cells revealed only the expression of MOCS1A, and no expression of the downstream MOCS1B ORF was observed (Fig. 4A). Gray and Nicholls \textsuperscript{(14)} presented further evidence that MOCS1B should not be expressed from the bicistronic construct; their phylogenetic com-

\textsuperscript{4} P. Hänzelmann, unpublished results.
\textsuperscript{5} J. Reiss, unpublished results (cited in Ref. 3).
Comparison of MOC1 sequences from different vertebrates and invertebrates revealed (i) no conserved initiation codon for MOC1B as well as (ii) no putative Kozak sequence. Together with our expression analysis, we conclude that MOC1B is only expressed as a MOC1A-MOC1B fusion protein from monocistronic mRNAs. Besides a catalytically active MOC1A protein, a multidomain protein incorporating both MOC1A and MOC1B may offer many benefits like enhanced reaction kinetics and coordinated regulation. Although the MOC1A domain is not active in the fusion proteins, it might be possible that partial activities like substrate/product and cofactor binding or interaction with MOC1B are unaffected. If MOC1A is able to form multimers, one can argue that heteromultimers are formed between MOC1A and MOC1A-MOC1B fusion proteins that would facilitate the interaction of catalytically active MOC1A and MOC1B. For example, from some SAM proteins it is known that they act only as a dimer, resulting in the stabilization of the FeS clusters (44, 45). Dimerization of MOC1A would then facilitate the proposed interaction between the C-terminal double glycine motif and the active site of MOC1B.

Expression of MOC1A in E. coli moaA mutants containing only wild type levels of endogenous E. coli MocaC resulted in a significant accumulation of precursor Z (Fig. 2). On the other side, MOC1B, which is also capable of catalyzing together with endogenous MocaA the formation of precursor Z, increased the level of precursor Z only slightly (Fig. 2). MOCS1A with its FeS cluster, probably its SAM and the reactive C-terminal double glycine motif, might by the catalytic part of the MOCS1A and MOC1B protein complex be regulating precursor Z synthesis via its expression level. Therefore, possible functions of MOC1B in precursor Z synthesis could be that (i) MOC1B might serve as a scaffold for the formation of precursor Z facilitating the proposed rearrangement reaction catalyzed by MOCS1A or (ii) MOC1B is the carrier protein for the oxygen-sensitive precursor Z that delivers precursor Z to MPT synthase for the subsequent formation of MPT.

Taken together, the involvement of C-terminal double glycine motifs in different biological processes is well known. Besides functions like in the ubiquitin-dependent protein degradation (40) or in sulfur transfer pathways (thiamine and MPT) (23, 39), we could identify a new type of function for this motif in MOC1A in precursor Z biosynthesis. In humans, this step of Moco biosynthesis needs the concerted action of proteins encoded by mono- and bicistronic mocs1 transcripts derived by alternative splicing.

Acknowledgments—We thank J. Reiss (Institute of Medical Physics and Biophysics, University of Münster, Germany) for providing the mocs1 splice type I cDNA and T. Giesemann (Zoological Institute, Technical University of Braunschweig, Germany) for help with transcription experiments. The technical assistance of T. Otte is gratefully acknowledged.

REFERENCES

1. Rajagopalan, K. V., and Johnson, J. L. (1992) J. Biol. Chem. 267, 10199–10202
2. Kisker, C., Schindelin, H., Baas, D., Retey, J., Meckenstock, R. U., and Kromek, P. M. (1998) FEMS Microbiol. Rev. 22, 503–521
3. Reiss, J. (2000) Hum. Genet. 106, 157–163
4. Johnson, J. L., Rajagopalan, K. V., and Wadman, S. K. (1993) Adv. Exp. Med. Biol. 338, 373–378
5. Reiss, J., Christensen, E., Kurlemann, G., Zabot, M. T., and Dorche, C. (1998) Hum. Genet. 103, 639–644
6. Reiss, J., Cohen, N., Dorche, C., Mandel, H., Mendel, R. R., Stallmeyer, B., Zabot, M. T., and Dierks, T. (1998) Nat Genet. 20, 51–53
7. Wuebbens, M. M., and Rajagopalan, K. V. (1995) J. Biol. Chem. 270, 1082–1087
8. Rieder, C., Eisenreich, W., O’Brien, J., Richter, G., Gotze, E., Boyle, P., Blanchard, S., Bach, A., and Simon, H. (1998) Eur. J. Biochem. 253, 35–36
9. Stallmeyer, B., Druegen, G., Reiss, J., Haenni, A. L., and Mendel, R. R. (1999) Am. J. Hum. Genet. 64, 698–705
10. Stallmeyer, B., Schwarz, G., Schulze, J., Nerlich, A., Reiss, J., Kirsch, J., and Mendel, R. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1333–1338
11. Feng, G., Tintrup, H., Kirsch, J., Nichol, M. C., Kuhne, J., Betz, H., and Sannes, J. R. (1998) Science 282, 1321–1324
12. Johnson, J. L., Wuebbens, M. M., Mandell, R., and Shih, V. E. (1999) J. Clin. Invest. 83, 897–903
13. Reiss, J., Gross-Haebt, S., Christensen, E., Schmidt, P., Mendel, R. R., and Schwarz, G. (2001) Am. J. Hum. Genet. 68, 208–213
14. Gray, T. A., and Nicholls, R. D. (2000) FEMS Microbiol. Lett. 190, 369–374
15. Unkles, S. E., Smith, J., Kanan, G. J., Millar, I. L., Heck, I. S., Bosher, D. H., and Kinghorn, J. R. (1997) J. Biol. Chem. 272, 28381–28390
16. Rivers, S. L., McNaair, E., Blasco, F., Giordano, G., and Bosher, D. H. (1993) Mol. Microbiol. 8, 1071–1081
17. Hoff, T., Schwenr, K. M., Meyer, C., and Caboche, M. (1999) J. Biol. Chem. 274, 6100–6107
18. Menendez, C., Igloi, G., Henningher, H., and Bransch, R. (1995) Arch. Microbiol. 164, 142–151
19. Menendez, C., Siebert, D., and Bransch, R. (1996) FEBS Lett. 391, 101–103
20. Solomon, P. S., Shaw, A. L., Lane, I., Hanson, G. R., Palmer, T., and McEwan, A. G. (1999) Microbiologica 145, 1421–1429
21. Sofia, H. J., Chen, G., Hetzler, B. G., Reyes-Spindola, J. F., and Miller, N. E. (2000) Nucleic Acids Res. 28, 1097–1106
22. Pitterle, D. M., Johnson, J. L., and Rajagopalan, K. V. (1993) J. Biol. Chem. 268, 15306–15309
23. Guzik, G., Fischer, B., Mendel, R. R., and Schwarz, G. (2001) J. Biol. Chem. 276, 36268–36274
24. Wuebbens, M. M., Liu, M. T., Rajagopalan, K. V., and Schindelin, H. (2000) Struct. Fold. Des. 6, 709–718
25. Brown, G. M. (1985) in Polylates and Pterins (Blakley, R. L., and Benkovics, S. J., eds) Vol. 2, pp. 299–419, John Wiley & Sons, Inc., New York
26. Bachar, A. (1990) in Chemistry and Biochemistry of Flavoenzymes (Müller, F., ed) Vol. 1, pp. 215–259, CRC Press, Inc., Boca Raton, FL
27. Casadaban, M. J. (1976) J. Mol. Biol. 104, 541–555
28. Johnson, M. E., and Rajagopalan, K. V. (1987) J. Bacteriol. 169, 117–125
29. Reiss, J., Kleinhub, A., and Klincmüller, W. (1987) Mol. Gen. Genet. 206, 352–355
30. MacGregor, C. H., Schmittman, C. A., and Normansell, D. E. (1974) J. Biol. Chem. 249, 5321–5327
31. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
32. Johnson, M. E., and Rajagopalan, K. V. (1987) J. Bacteriol. 169, 110–116
33. Schwarz, G., Boxer, D. H., and Mendel, R. R. (1997) J. Biol. Chem. 272, 26811–26814
34. Graham, F. L., and van der Ebb, A. J. (1973) Virology 91, 456
35. Baker, K. P., and Boxer, D. H. (1991) Mol. Microbiol. 5, 901–907
36. Rudolph, M. J., Wuebbens, M. M., Rajagopalan, K. V., and Schindelin, H. (2001) Nat. Struct. Biol. 8, 42–46
37. Leimkuhler, S., Wuebbens, M. M., and Rajagopalan, K. V. (2001) J. Biol. Chem. 276, 34695–34701
38. Lake, M. W., Wuebbens, M. M., Rajagopalan, K. V., and Schindelin, H. (2001) Nature 414, 325–329
39. Begley, T. F., Xi, J., Kinsland, C., Taylor, S., and McLafferty, F. (1999) Curr. Opin. Chem. Biol. 3, 623–629
40. Hershko, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807
41. Wuebbens, M. M., and Rajagopalan, K. V. (1993) J. Biol. Chem. 268, 13493–13498
42. Rajagopalan, K. V. (1996) in Escherichia coli and Salmonella typhimurium (Neidhardt, F. C., ed) pp. 674–679, ASM Press, Washington, D. C.
43. Pitterle, D. M., and Rajagopalan, K. V. (1993) J. Biol. Chem. 268, 13499–13505
44. Cheek, J., and Broderick, J. B. (2001) J. Biol. Inorg. Chem. 6, 209–226
45. Frey, P. A. (2001) Annu. Rev. Biochem. 70, 121–148
Functionality of Alternative Splice Forms of the First Enzymes Involved in Human Molybdenum Cofactor Biosynthesis
Petra Hänzelmann, Günter Schwarz and Ralf R. Mendel

J. Biol. Chem. 2002, 277:18303-18312. 
doi: 10.1074/jbc.M200947200 originally published online March 12, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M200947200

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

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

This article cites 44 references, 17 of which can be accessed free at http://www.jbc.org/content/277/21/18303.full.html#ref-list-1