**Mep1 Encodes the Molybdenum Cofactor Carrier Protein in Chlamydomonas reinhardtii and Participates in Protection, Binding, and Storage Functions of the Cofactor**

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**Farid Shokry Ataya, Claus Peter Witte‡, Aurora Galván, María Isabel Igeño§, and Emilio Fernández¶**

From the Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, Campus de Rabanales, Edificio Severo Ochoa, Córdoba 14071, Spain

The molybdenum cofactor (Moco) is essential for the activity of all molybdoenzymes except nitrogenase. The cDNA for the Moco carrier protein (MocoCP) of *Chlamydomonas reinhardtii* has been cloned by reverse transcription PCR approaches with primers designed from microsequenced peptides of this protein. The *C. reinhardtii* MocoCP has been expressed in *Escherichia coli*. The recombinant protein has been purified to electrophoretic homogeneity and is found assembled into a homotetramer when Moco is not present under native conditions. Recombinant MocoCP has the same biochemical characteristics as MocoCP from *C. reinhardtii*, as it bound Moco from milk xanthine oxidase with high affinity, prevented Moco inactivation by oxygen, and transferred Moco efficiently to aponitrate reductase from the *Neurospora crassa* nit1 mutant. The genomic DNA sequence corresponding to the *Chlamydomonas* MocoCP gene, CrMcp1, also was isolated. This gene contained three introns in the coding region. The deduced amino acid sequence of CrMcp1 did not show a significant identity to functionally known proteins in the GenBank™ data base, although a significant conservation was found with bacterial proteins of unknown function. The results suggest that proteins having a Moco binding function probably exist in other organisms.

In eukaryotes, eubacteria, and archaeabacteria, all molybdoenzymes carry Moco in their active sites and share the core structure. Moco consists of molybdopterin, an alkylated, not fully aromatic pterin, complexing one molybdenum atom via a dithiolene group to its four-carbon side chain. It occurs in several dihydroforms, depending on the enzyme, that facilitate the oxidation-reduction reaction (1). In prokaryotes, the cofactor can be modified by an additional nucleotide monophosphate bound to the C-4’ of the phosphogroup within the alkylic side chain of the pterin (2, 3).

Molybdoenzymes are widespread and essential for diverse metabolic processes such as the first step of nitrate assimilation in autotrophs, sulfur detoxification and purine catabolism in mammals, and phytohormone synthesis in plants (4–6). Thus the survival of many living organisms depends on their ability to synthesize Moco and to maintain it in a preserved active form. Several diseases have been related to a defect in Moco synthesis (7). Among the diverse organisms studied, at least six gene products are involved in the formation of active Moco (1, 2). Moco biosynthesis occurs by a multistep reaction of molybdopterin synthesis followed by the incorporation of molybdenum (2).

Moco activity is routinely assayed by measuring the reconstituted nitrate reductase activity from the inactive apoenzyme of the *Neurospora crassa* fungal mutant strain nit1, which lacks active Moco (8). This cofactor is very unstable in vitro, and molybdenum readily dissociates from the cofactor complex, converting irreversibly into an inactive form such that molybdate stabilizes Moco (9, 10). Other sulfhydryl-protecting agents stabilize Moco (10, 11), whereas sulfhydryl-reactive inhibitors prevent the reconstituting activity of the cofactor (12), indicating the participation of free sulfhydryl groups in the reconstitution process. Oxygen seems to be a major factor of free Moco inactivation, which might be especially dramatic in autotrophs because of the photosynthetic activity. Thus the presence of a stabilizing agent in vivo appears to be essential.

Information on the storage of active Moco in the cells is scarce. In a previous study (13), we purified a Moco-binding protein from *Chlamydomonas reinhardtii*. It was named molybdenum cofactor carrier protein (MocoCP) because of its ability to transfer Moco directly to aponitrate reductase (14). This 64-kDa MocoCP with four identical subunits of 16.5 kDa protected Moco against inactivation under aerobic conditions and basic pH levels. Proteins with similar characteristics were also found in *Vicia faba* (15) and *Escherichia coli* (16).

In this work, we present the first molecular evidence for the presence of a MocoCP in eukaryotes. The recombinant MocoCP binds Moco with a high affinity and stabilizes Moco against in vitro inactivation by oxygen.

**MATERIALS AND METHODS**

**Strains and Growth Conditions—** *C. reinhardtii* wild type has been characterized elsewhere (17). Cells were grown at 25 °C under continuous illumination in a liquid minimal medium that contained 4 mM KNO₃ as a nitrogen source bubbled with 4% (v/v) CO₂-enriched air (18).
Cloning of Gene Encoding Moco Carrier Protein

N. crassa nit1 mutant (Fungal Genetic Stock Center, Arcata, CA) was grown in an ammonium-containing medium and induced in 20 mM nitrate under previously reported conditions (19). Unless otherwise stated, all E. coli strains were grown in LB medium supplemented with 50 μg/ml ampicillin, 25 μg/ml kanamycin, or both. Where appropriate, E. coli strains LE392 and BM25.5 were used for library screening and automatic subcloning, respectively. Further subcloning of cDNA or genomicDNAs was performed using E. coli strains DH5α or XL1 Blue. Expression of recombinant MocoCP was carried out in the E. coli strain M15.

Microsequencing of MocoCP—MocoCP was purified as reported previously (13) and subjected to a peroxidase-catalyzed reaction (20). The protein was blocked onto a polyvinylidene difluoride membrane and visualized with Computerized Bright Blue R-250 stain (0.1% w/v). The amino-terminal sequence was generated from the protein band cut from the dry blot. MocoCP was digested with CNBr after gel electrophoresis (21). The equilibrated gel pieces were subjected to a second electrophoresis using Tricine-SDS-PAGE (22). The separated peptides were blocked on a polyvinylidene difluoride membrane and processed like the amino-terminal blocked protein. Sequencing was carried out at the Gesellschaft für Biotechnologische Forschung (GBF, Braunschweig, Germany) on a gas-phase sequencing apparatus. Two sequences were obtained for peptides from CNBr digestion: GPGKADTAENQLVMANELGKQIATHG and MGPFTAAEVALAKKPPVLV.

Oligonucleotide Design, cDNA Synthesis, and Reverse Transcription PCR—Two degenerate primers corresponding to PGKATDR and MGPFTAAEVA were designed from the microsequenced protein of purified C. reinhardtii MocoCP by employing the deduced codon usage for C. reinhardtii genes. These primers are named MOP1 (forward, 5'-CCCGGCAAGGCCGACCANGCNGAR-3') and MOP2 (reverse, 5'-GCCACCTCGGCGGCGGTNCCNGGNCCCAT-3'), respectively, where N is any of the four nucleotides and R corresponds to A or G. These primers were used in reverse transcription PCR for amplification of a MocoCP cDNA fragment. On the other hand, new primers were designed for amplifying the complete coding region of the MocoCP gene for expression in E. coli. New restriction sites for XbaI and EcoRI were included in the forward (MF1, 5'-TCTAGACATGTCGGGACGAAGC-3') and reverse (MR1, 5'-CTACCGTTAGCAGAAGAAAC-3') primers, respectively, to facilitate further subcloning in the expression vector. The sense primer contained the start codon in frame and the antisense primer contained the stop codon.

cDNA synthesis was performed with 1 μg of total RNA extracted from the wild type 21g of C. reinhardtii cultivated in 4 ml nitrate by reverse transcription amplification using the Superscript™ II kit (Invitrogen). Reverse transcription PCR was performed with Taq DNA polymerase (BioTools B&M Laboratories) in reactions containing 2.5% MeSO4, as recommended by the manufacturer, with the following cycling conditions: 94°C for 1 min, then 40 cycles at 94°C for 30 s, 65°C for 1 min. The selected PCR fragment was cloned into the pGEM-T vector (Promega).

Screenings of C. reinhardtii Libraries and Subcloning—The C. reinhardtii cDNA library in AEXeo (kindly provided by Dr. Paul Lefebvre, University of Minnesota, St. Paul, MN) was propagated and blotted onto nylon filters. Subsequent hybridization was performed (23) using a DNA probe randomly labeled by digoxigenin (Roche Molecular Biochemicals), and positive signals were detected with alkaline phosphatase-conjugated antidigoxigenin antibodies and the chemoluminescent DNA probe randomly labeled by digoxigenin (Roche Molecular Biochemicals). N. crassa nit1 mutant as described previously (8) was propagated and blotted of cDNA randomly labeled by digoxigenin (Roche Molecular Biochemicals). The genomic library in tase-conjugated antidigoxigenin antibodies and the chemoluminescent DNA probe randomly labeled by digoxigenin (Roche Molecular Biochemicals) was propagated and blotted on nylon filters. cDNA synthesis was performed with 1 μg of total RNA extracted from the wild type 21g of C. reinhardtii cultivated in 4 ml nitrate by reverse transcription amplification using the Superscript™ II kit (Invitrogen). Reverse transcription PCR was performed with Taq DNA polymerase (BioTools B&M Laboratories) in reactions containing 2.5% MeSO4, as recommended by the manufacturer, with the following cycling conditions: 94°C for 1 min, then 40 cycles at 94°C for 30 s, 65°C for 1 min. The selected PCR fragment was cloned into the pGEM-T vector (Promega).

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FIG. 1. Nucleotide and deduced amino acid sequences of the CrMcpl gene. Mcpl sequence (GenBank™ accession number Y039706) shows the promoter region, 4 exons (capital letters) separated by 3 introns (lowercase letters), and the 3'-untranslated region. Two putative nitrate-controlled signals are indicated in bold letters in the promoter sequence. The nucleotide sequence for the cDNA (GenBank™ accession number Y039707) is shown as underlined letters at the 5' - and 3'-untranslated regions, and the coding sequence is shown in capital letters. Two overlapping polyadenylation signals (bold, underlined letters) in the 3'-untranslated region are also indicated.
and incubated at 37°C with shaking for 3 h. The cells were harvested by centrifugation and resuspended in 10 ml of the PBST extraction buffer (10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl, 1% Triton X-100) containing 2 mM EDTA, 0.1% (v/v) β-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride. The cells were lysed by ultrasonication, and the bacterial lysate was centrifuged at 10,000 x g for 10 min to remove the cell debris. The clear crude extract was applied onto a GSH-agarose column (0.5 to 1 ml), after washing the matrix with PBST, the fusion protein was eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM GSH. The eluted buffer. After washing the matrix with PBST, the fusion protein was eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM GSH. The eluted fraction was concentrated by centrifugation in Centricon (Millipore) and washed with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2, and 0.1% (v/v) β-mercaptoethanol. 50 units of thrombin were added for 4 h at room temperature to digest the link between GST and MocoCP. The digested protein was applied onto a minicolumn of Sephadex G-25 to eliminate GSH, and the high molecular weight fraction was subject to rechromatography in GSH-agarose. The eluted fractions contained the purified protein were determined from the UV light absorption at 280 nm corresponding to the single tryptophan residue of MocoCP. The purity of the protein was judged from SDS-PAGE (28) with 15% separation gel after visualizing the protein bands with Coomassie Brilliant Blue R-250. The molecular mass was calculated from migrations of molecular weight markers (Sigma). The concentrations of the purified protein were determined from the UV light absorption at 280 nm corresponding to the single tryptophan residue of MocoCP (ε = 5.6 M⁻¹ cm⁻¹).

RESULTS AND DISCUSSION
Cloning and Characterization of the MocoCP cDNA and the MocoCP Gene (CrMcp1) from C. reinhardtii—A cDNA fragment of 300 bp was amplified by reverse transcription PCR using the primers MOP1 and MOP2. They were designed from protein microsequencing of purified MocoCP from C. reinhardtii and its CNBr digestion peptides. The DNA sequence of this cDNA fragment included both of the primers used and additional amino acid residues present in the original protein microsequencing, which indicated that the correct fragment had been amplified. This 300-bp fragment was used as a probe to screen a cDNA library from C. reinhardtii 21gr cells grown in nitrate medium. From about 80,000 phages screened, 7 hybridizing phages were identified, recovered, and subcloned into the pEX-lox vector. The cloned plasmids released insertions of 0.8–1.0 kb after EcoRI and HindIII digestions. The cDNA sequencing revealed an open reading frame of 495 nucleotides encoding a protein of 165 residues with a calculated molecular mass of 16.5 kDa. The cDNA also defined 45 nucleotides at the 5'-untranslated region and 446 at the 3'-untranslated region, which has two possible noncanonical and overlapping polyadenylation signals, TGTCAGTAA (Fig. 1), as compared with the typical signal in Chlamydomonas, and TGTTA (29). A similar situation of contiguous noncanonical polyadenylation signals was found in the C. reinhardtii Nar1 gene (30).

The full-length MocoCP cDNA was used as a probe to screen about 60,000 phages from a genomic library in λEMBL4. Nine clones with the highest signals were chosen for further study. These phages corresponding to the C. reinhardtii MocoCP gene (CrMcp1) released inserts with an average length of 5–12 kb by EcoRI digestion as indicated by Southern blot analysis. One of these fragments of about 6 kb was cloned in Bluescript KS (+/−) and sequenced. The DNA sequencing of the genomic clone revealed an open reading frame of 495 nucleotides similar
TABLE I
Cloning of Gene Encoding Moco Carrier Protein

| Sample                  | Moco activity in the excluded fraction | Moco activity in the included fraction |
|-------------------------|---------------------------------------|---------------------------------------|
| GST-MocoCP              | ND                                    | ND                                    |
| MocoCP                  | ND                                    | ND                                    |
| GST-MocoCP + Moco       | 2 ± 3                                 | 95 ± 3                                |
| MocoCP + Moco           | 98 ± 5                                | 5 ± 4                                 |

TABLE II
Efficiency of recombinant MocoCP to bind Moco extracted from milk XO

| Sample                  | Incubation time | Excluded fraction % | Included fraction % |
|-------------------------|-----------------|---------------------|---------------------|
| Free Moco               | 0               | 0                   | 100                 |
| Free Moco + MocoCP      | 0               | 95 ± 3              | 4 ± 1               |
| Free Moco + MocoCP      | 5               | 94 ± 5              | 2 ± 1               |
| Free Moco + MocoCP      | 10              | 98 ± 4              | 3 ± 1               |

The comparison of the deduced MocoCP amino acid sequence with sequences from the GenBankTM showed similarity to proteins with unknown functions. The sequences with the highest conservation were from bacteria and Archaea (Fig. 2A). The maximum similarity of 49.7% was found with a putative protein from the cyanobacterium Trichodesmium erythraeum (GenBankTM accession number ZP_00073012). Moderate identities were also found with a putative protein deduced from Aquifex aeolicus (31.1%, GenBankTM accession number AAC06500) and with predicted Rossmann fold nucleotide-binding proteins from Archaea Methanopyrus kandleri (33.1%, GenBankTM accession number NP_614673) and Crenarchaeota 74A4 (27.9%, GenBankTM accession number AAK96093). A limited and insignificant identity was found with protein sequences from plants in the GenBankTM data base. The phylogenetic tree of the examined proteins (Fig. 2B) shows that the cyanobacterial protein is grouped together with the C. reinhardtii MocoCP, both of which are at a similar distance from the proteins from M. kandleri and A. aeolicus, all of which are more distantly related to the protein from the Crenarchaeota sp. Although no protein from plants showed conservation with MocoCP, it is interesting to point out that there is a protein with functionality of the Moco carrier in V. faba (15).

The molecular analysis of the 165-amino acid sequence of MocoCP using the program PROTEAN showed that this protein is highly hydrophobic. The calculated pl of 6.1 is higher than that of 4.5 determined experimentally for native MocoCP purified from C. reinhardtii (13). This difference could be due to the tetrameric form of the native protein, which may affect the net charge of the whole molecule. All examined molybdenoenzymes, such as NR from various sources (32, 33), sulfite oxidase of rat liver (34, 35), XO, and aldehyde oxidase (36), showed an invariant Moco-binding site in the Moco domain with the characteristic signature sequence CAGNNR. This sequence is suggested to be involved in Moco binding, and a mutation in the C residue completely abolishes the NR and sulfite oxidase activity of Pichia (33, 37). However, the MocoCP-deduced amino acid sequence does not contain any C residue nor, subsequently, does it contain the signature sequence CAGNR.

Thus, the Moco-binding site in MocoCP seems to differ from that in the Moco domain of molybdenoenzymes by allowing both efficient binding and release of Moco.

Southern blot analysis of digested genomic DNA from the C. reinhardtii wild type 6145c using the cDNA as a probe showed hybridization bands compatible with a single CrMcp1 gene copy (data not shown).

Little is known about the regulation of Moco genes in eukaryotes. In plants, Moco gene expression was found to be extremely low so that standard Northern blots hardly give signals. As expected for housekeeping genes, Moco genes are expressed constitutively at a basal level (38). The CrMcp1 gene seems to belong to this family of genes, as it barely gave a signal using standard Northern blots (data not shown). The availability of sufficient amounts of Moco in a constitutive form is essential for the cell to meet its changing demand for synthesizing particular enzymes. The multistep synthesis of Moco and the low expression together with the instability of synthesized Moco suggest that the existence of Moco storage proteins would be a good means by which to buffer the supply and demand of Moco.

Expression and Functionality of the Recombinant MocoCP in E. coli—The MocoCP from C. reinhardtii was expressed in E. coli that had been transformed with the expression vector pGEX, which contained the full coding region of MocoCP. Purification of the GST-MocoCP fusion protein had been per-

![Fig. 4. Protection of Moco activity by recombinant MocoCP.](image-url)
formed by specific elution with glutathione in a GSH-agarose affinity chromatography. After digestion with thrombin, GST and MocoCP were separated by rechromatography on the same affinity column. MocoCP eluted directly in the flowing fractions and showed a high homogeneity as judged by SDS-PAGE (Fig. 3). The recombinant MocoCP showed a molecular mass higher than that of the predicted protein (16.5 kDa) given that digestion by thrombin leaves 14 amino acids from the linker region of the fusion protein (GSGGSGGGGGGGLD). This glycine-rich linker facilitates the proteolytic digestion by thrombin (27). As shown below, the recombinant MocoCP has a high binding affinity to Moco and transfers Moco efficiently to the apoenzyme from N. crassa nit1 extracts, indicating that this linker did not affect the function of MocoCP.

In C. reinhardtii, Moco exists in two main forms, bound to MocoCP and bound to molybdooenzymes, as free Moco is hardly detectable (40). Moco bound to MocoCP in C. reinhardtii is unable to reconstitute the NR activity of the N. crassa nit1 mutant when separated by a dialysis membrane, whereas free Moco extracted from XO can achieve reconstitution. A direct contact between the MocoCP charged with Moco and the apoNR of N. crassa was proposed for the reconstitution of the enzyme activity (13, 14). Recombinant MocoCP was subjected to molecular exclusion chromatography under native conditions. MocoCP behaved as a protein with a 70-kDa global molecular mass, which corresponded to a tetramer, whereas the fusion protein GST-MocoCP appeared to assemble into dimers of 90 kDa and multimers of more than 280 kDa (data not shown). In the absence of added Moco, both recombinant MocoCP and MocoCP bound to GST did not contain bound Moco as determined by the nit1 reconstitution assay (Table I). However, after incubation with Moco from milk XO, recombinant MocoCP bound Moco but not the MocoCP-GST fusion protein. These results suggest that Moco is not required for the assembly of subunits in the quaternary structure of MocoCP and that GST interferes with the appropriate folding of MocoCP to bind Moco.

The ability of the recombinant MocoCP to bind and protect Moco against inactivation by oxygen was also assayed by the nit1 complementation assay. Free Moco was released from milk XO by heat treatment under anaerobic conditions, and its binding to recombinant MocoCP was determined at different time intervals under aerobic conditions after filtering the binding mixtures through a spin column. Both free Moco and Moco bound to MocoCP were determined in the included and excluded fractions, respectively. Interestingly, recombinant MocoCP was able to bind free Moco from XO almost instantaneously (Table II). Thus recombinant MocoCP shows an extremely high affinity to bind free Moco from XO.

Recombinant MocoCP protected Moco against inactivation by oxygen. When bound to recombinant MocoCP, Moco was stabilized against this inactivation. Free Moco had lost its activity after 5 h of incubation in aerobic conditions (Fig. 4), whereas Moco bound to recombinant MocoCP was still more than 93% active under the same conditions. Even after 2 h in the presence of oxygen, MocoCP conserved 30% of its Moco activity (Fig. 4). In photosynthetic organisms, large amounts of oxygen are produced during photosynthesis. This oxygen would inactivate intracellular free Moco, if present. In C. reinhardtii, the presence of many light-controlled sites in the promoter of CrMcp1 would ensure a harmonious induction of this protein with the production of photosynthetic oxygen. The high affinity binding of Moco, the low concentration of free Moco, and the necessary contact of MocoCP and apoNR to transfer Moco (39) together suggest that MocoCP functions as a binding, storage, protective, and carrier protein. As with all cellular molecules, molybdooenzymes degrade through normal protein turnover after achieving their catalytic function. There is no information about the fate of Moco. The low level of Moco biosynthesis and the high binding affinity of the MocoCP also suggest a role for MocoCP in the recycling of Moco. Further studies are needed to clarify the role of MocoCP in living organisms.

Conclusions—The isolation of the cDNA and genomic DNA for the C. reinhardtii MocoCP provides a definitive molecular support for the existence of this protein in eukaryotes. The CrMcp1 gene encodes a protein that contains the sequence of peptides microsequenced from purified C. reinhardtii MocoCP. In addition, the protein expressed from cDNA constructs in E. coli is functionally active and demonstrates the properties of Moco binding and Moco protection shown for the protein in the alga. Proteins related to MocoCP from C. reinhardtii appear to be present in cyanobacteria and other bacteria, although a clear sequence conservation does not appear to exist with other eukaryotic proteins. Our data show that, in Chlamydomonas, Mcp1 encodes MocoCP, a homotetrameric protein that binds Moco with a high affinity and plays a key role in the maintenance and storage of Moco in an active form under aerobic conditions.

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