Function and Structure of the Molybdenum Cofactor Carrier Protein from *Chlamydomonas reinhardtii*

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Katrin Fischer†§1, Angel Llamas†§1, Manuel Tejada-Jimenez†§1, Nils Schrader‡, Jochen Kuper**, Farid S. Ataya†, Aurora Galvan§, Ralf R. Mendel§, Emilio Fernandez§, and Guenter Schwarz‡§2

From the †Institute of Plant Biology, Technical University Braunschweig, 38106 Braunschweig, Germany, the §Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, Córdoba 14071, Spain, the ¶Institute of Biochemistry, University of Cologne, 50674 Cologne, Germany, and the **European Molecular Biology Laboratory (EMBL) outstation, 22603 Hamburg, Germany, and the ¶¶Institute of Molecular Physiology, 44247 Dortmund, Germany, the European Molecular Biology Laboratory (EMBL)

The molybdenum cofactor (Moco) forms the catalytic site in all eukaryotic molybdenum enzymes and is synthesized by a multistep biosynthetic pathway. The mechanism of transfer, storage, and insertion of Moco into the appropriate apo-enzyme is poorly understood. In *Chlamydomonas reinhardtii*, a Moco carrier protein (MCP) has been identified and characterized recently. Here we show biochemical evidence that MCP binds Moco as well as the tungstate-substituted form of the cofactor (Wco) with high affinity, whereas molydbodopterin, the ultimate cofactor precursor, is not bound. This binding selectivity points to a specific metal-mediated interaction with MCP, which protects Moco and Wco from oxidation with t1/2 of 24 and 96 h, respectively. UV-visible spectroscopy showed defined absorption bands at 393, 470, and 570 nm pointing to one di-thiolate and protein side-chain charge transfer bonds with molybdenum. We have determined the crystal structure of MCP at 1.6 Å resolution using seleno-methionated and native protein. The monomer constitutes a Rossmann fold with two homodimers forming a symmetrical tetramer in solution. Based on conserved surface residues, charge distribution, shape, in silico docking studies, structural comparisons, and identification of an anion-binding site, a prominent surface depression was proposed as a Moco-binding site, which was confirmed by structure-guided mutagenesis coupled to substrate binding studies.

Molybdenum is used in the active center of all molybdenum enzymes (1), catalyzing key metabolic reactions in the global sulfur, nitrogen, and carbon cycles in organisms ranging from bacteria to human. With the exception of nitrogenase, in all molybdenum enzymes, molybdenum is found as molybdenum cofactor (Moco) (2) that consists of molybdenum covalently bound to the di-thiolate moiety of a tricyclic pterin referred to as molydbodopterin or metal-binding pterin (MPT), whose structure is conserved in eukaryotes, eubacteria, and archaea (3). This pterin molecule is also responsible for metal chelation in all tungsten-containing enzymes analyzed so far. Molybdenum enzymes are essential for diverse metabolic processes such as nitrate assimilation in autotrophs and phytohormone synthesis in plants (4) or sulfur detoxification and purine catabolism in mammals (5). Loss of Moco results in the pleiotropic loss of all molybdenum enzymes. Human Moco deficiency is a severe hereditary metabolic disorder (6), and affected patients die in early childhood.

In all organisms studied so far, Moco is synthesized by a conserved pathway that can be divided into four major steps (3), according to the biosynthetic intermediates cyclic pyranopterin monophosphate, (formerly described as precursor Z) (7), MPT, and adenylated MPT. In the final and most diverse step of Moco biosynthesis, a single molybdenum atom is ligated to one (in pro- and eukaryotes) or two MPT dithiolates (in prokaryotes).

After completion of biosynthesis, mature cofactor has to be inserted into molybdenum enzymes. In prokaryotes, a complex of proteins synthesizing the last step(s) of Moco biosynthesis was proposed to donate the mature cofactor to apo-enzymes (8) assisted by enzyme-specific chaperones (9). In eukaryotes, no molybdenum enzyme-specific chaperone has been found until now, and direct transfer of Moco is possible at least in *vitro* (10). However, in several organisms such as *Escherichia coli* (11), plant seeds (12) and the green alga *Chlamydomonas reinhardtii* (13), Moco-binding proteins were found. Due to the two known classes of eukaryotic molybdenum enzymes (1), differences in the insertion of Moco might also be considered. Enzymes of the sulfite oxidase (SO) family are characterized by a highly conserved cysteine residue (14, 15) providing a third sulfur atom in the square-pyramidal geometry of the molybdenum center. In contrast, enzymes of the xanthine oxidoreductase family contain a terminal sulfide group as third sulfur ligand (16), and so...
Structure of the Molybdenum Cofactor Carrier Protein

Cloning, Expression, and Purification of MCP—CrMCP1 (20) was PCR-cloned into BamHI and HindIII sites of pQE80 yielding pQE80-CrMCP1. To obtain MCP saturated with MPT and/or Moco, we transformed pQE80-CrMCP1 into E. coli strains RK5206 (mogA/H9262), or BL21. MCP was expressed for 36 h at 22–37 °C in LB medium containing 500 mg/l of seleno-methionine. For crystallization, MCP-bound Moco was incubated with nit-1 crude extract for 30 min to 21 h at room temperature followed by the determination of reconstituted NADPH-NR activity. Nitrite formed in a final volume of 125 μl was quantified by the absorbance at 540 nm using a 96-well plate reader (Vera max, Molecular Devices). One unit of Moco activity was defined as reconstituted nit-1 NR activity sufficient to produce an increase of 1.0 absorbance units at 540 nm per 25 min of reaction time. For kinetic studies of Moco transfer, 55–2000 fmol of Moco (bound to MCP) were used and incubated with nit-1 crude extract for 30 min to 21 h at room temperature. Reconstituted NR-activity was determined after 10 min of reaction time.

Crystallization and Structure Determination—MCP crystals were obtained from 16% polyethylene glycol 4000, 150 mM sodium acetate, and 100 mM Tris/HCl, pH 8.0, using hanging drop vapor diffusion. Se-Met crystals diffracted X-rays up to 2.4 Å at the synchrotron beamline BL1 at Berliner Elektronen-speicherring-Gesellschaft fuer Synchrotronstrahlung (BESSY), Berlin, Germany (see Table 1, MCP1). Native crystals diffracted up to 1.6 (MCP2) and 2.3 Å (MCP3) resolution, using either synchrotron radiation (BL1@BESSY) or a rotating anode (Rigaku R4+), respectively. Data sets were integrated and scaled using the HKL2000 package (MCP1, MCP2) or MOSFLM (24) (MCP3) and SCALA from the CCP4 suite (25). Starting with MCP1, the substructure was solved using HYSS from the PHENIX package (26), the obtained phases were refined, and the phase problem was solved with SOLVE (27). The model was automatically built with RESOLVE (28) and refined by cycles of manual model building in O (29) or COOT (30) and refinement in REFMAC5 (31). Molecular replacement using PHASER (32) served for solving the native structures of MCP2 and MCP3. The high resolution of MCP2 allowed using the “autobuild” function of ARP/WARP (33). Further refinements were carried out as described before. Water molecules were found with COOT (30) and ARP/WARP (33). Atomic coordinates were deposited in the RCSB Protein Data Bank.

Docking Studies—Automated docking was performed using the “Darwinian Genetic Algorithm” of GOLD (version 3.0) (34, 35), which explores the full range of ligand conformational flexibility with partial flexibility of the protein. The scoring function allows us to include terms for hydrogen bonding, van der Waals, and intramolecular energies. Moco was docked to the putative binding pocket of each monomer of the three MCP crystal forms with O61 of Asp-93 as the flood fill center of a 20 Å docking sphere assuming one cavity. In all runs, operator weights for crossover, mutation, and migration were set to 95, 95, and 10, respectively, for a population size of 100 and a selection pressure of 1.1. The “early termination” option was selected, and the top three solutions for each docking run were analyzed. The solutions were superimposed, and their fit was evaluated regarding electrostatic characteristics as well as the shape of the surface and conservation of interacting residues.

RESULTS

Selective Binding of Moco and Wco to MCP—To characterize the substrate binding properties, we expressed MCP in differ-
saturations of up to 6% (Fig. 1A). To synchronize intracellular protein and cofactor synthesis, we reduced the expression temperature to 22 °C and lowered the expression level (50 nm isopropyl-β-thiogalactoside), resulting in 25% Moco saturation (Fig. 1B). We also replaced molybdate by tungstate (1 mm) and found an even higher co-purification of pterin (up to 75%), pointing to Wco binding as we have previously shown that MCP binds no MPT (Fig. 1A).

The identity of co-purified Moco was confirmed by the nit-1 reconstitution assay (19), which was performed to detect exclusively Moco (39). According to the amount of co-purified pterin, Moco activity was found that correlated with the amount of molybdate added to the culture medium (Fig. 1A). In summary, MCP binds Moco with high affinity as described earlier (18, 20), but this binding is highly specific to Moco and Wco because metal-free MPT is not bound.

UV-visible Absorption Spectra of Cofactor Saturated MCP—As MCP was purified highly saturated with either Moco or Wco, we applied UV-visible spectroscopy to analyze the metal centers. MCP saturated with Moco (25%) shows absorption at 393 nm and additional shoulders around 470 and 570 nm (Fig. 1C). Similar to plant (40) and animal SOs (41), the absorption at 393 nm can be attributed to the ene-dithiolate-to-molybdenum charge transfer bond. As MCP lacks any cysteine, the absorption bands at higher wavelengths might point to additional amino acid-to-molybdenum charge transfer bonds.

Upon aerobic incubation (12 h), all aforementioned bands are lowered, which is mainly due to degradation of Moco (see below). However, slight changes are observed, such as a more pronounced peak at 393 nm and shoulder at 470 nm (Fig. 1C, dotted line). The addition of excess dithionite (as compared with bound Moco) resulted in small but distinct changes in the spectrum. All three absorption bands are diminished upon reduction, resulting in a spectrum almost identical to the initial one, which indicates, together with the anaerobic purification of MCP, that Moco is co-purified in a reduced state and can be oxidized.

UV-visible spectra of Wco-loaded MCP do not show any pronounced profile in the range of 350–700 nm (Fig. 1D). Upon aerobic incubation, an overall reduction of absorption can be seen with a slight increase between 600 and 700 nm, whereas the addition of dithionite did not alter the spectrum significantly.

MCP Protects Moco and Wco from Oxidation—Next we investigated the stability of MCP-bound Moco and Wco under aerobic conditions (Fig. 2, A and B). For both ligands, temperature- and time-dependent degradation was found, with Moco degrading much faster than Wco at all temperatures examined. The half-lives of Moco were not much affected by reduced temperatures. In contrast, the stability of Wco at high temperatures was similar to Moco, whereas at 4 °C, nearly no degradation was found. This points to a tighter binding of Wco to MCP and might explain why Wco was able to co-purify with up to 75% saturation.
Moco Transfer from MCP to NR—High levels of co-purified Moco and Wco indicate high affinity binding to MCP, suggesting a Moco storage function. Thus we also wanted to know whether MCP transfers Moco (and also Wco) directly to the user enzyme or whether the ligand is released by dissociation and later bound to the enzymes. nit-1 reconstitutions with Moco-loaded MCP in the presence of increasing concentrations of tungstate (Fig. 2C) showed that only with 10 mM tungstate was Moco activity significantly inhibited, indicating rapid transfer between MCP and apo-NR. When using Wco-loaded (25%) MCP, no nit-1 activity was found according to the presence of the antagonistic metal tungsten. In contrast to the displacement of molybdenum by tungsten during Moco transfer, no reconstitution was found for Wco-bound MCP in the presence of external molybdate, demonstrating the tight binding of tungsten to MPT and/or Wco to MCP.

The competition by tungstate pointed to a rapid transfer of Moco to NR. Therefore, we investigated the kinetics of NR transfer between MCP and apo-NR. When using Wco-loaded MCP in the presence of increasing concentrations of the competing anions tungstate (C) and molybdate (D), similar cofactor saturations were used (25%). E and F, time-dependent nit-1 reconstitutions with MCP-bound Moco. Due to biphasic kinetics, the initial (E) and secondary (F) transfer rates are compared. The amounts of Moco-loaded MCP are indicated in each figure.

Structure of the Molybdenum Cofactor Carrier Protein

As shown previously, MCP forms stable tetramers in solution (18, 20). Although in the MCP1 structure, the tetramer presents the content of the asymmetric unit, in both native structures, tetramers are formed by crystalllographic symmetry. Within the tetramer, each monomer contributes to two distinct intermolecular interactions (Fig. 3B), resulting in the formation of dimers of dimers. Interface I is predominantly characterized by contacts involving helices α3 and α4 of each monomer and has rather hydrophobic character with 76–80% non-polar residues, and not more than one H-bond and eight bridging water molecules are found in this contact region. The other intermolecular contact (Fig. 3B, Interface II) is mainly formed by strands β2, β3, and β4, mediated by 30–42% polar residues, up to 105 bridging water molecules and eight H-bonds. All dimer interface layers cover a similar surface area (~725–875 Å²), and superposition of the tetramers shows high similarity, proving that the quaternary structure of MCP is not altered by the different crystal lattices and asymmetric units. Despite up to 245 water molecules in the crystals, we could not detect any ligand bound to MCP. Although MCP1 and...
FIGURE 3. Structure of C. reinhardtii MCP. A, ribbon presentation of the MCP monomer (MCP2 chain A) with β-strands colored in gold and α-helices shown in green. Residues Gly-70 and Ile-76 that border the stretch of delocalized residues are highlighted. B and C, top view (B) and side view (C) of the tetramer in ribbon presentation (MCP2). the color code of the monomers is as follows: A, red; B, green; C, blue; D, yellow. The monomers C and D were generated by applying crystallographic symmetry operations. Interface I depicts the hydrophobic contacts between A and B and C and D, and Interface II describes the more hydrophilic contacts of B and C and A–D. D, superposition of MCP2 (chain B, green) with T. thermophilus HB8 hypothetical protein TT1465 (Protein Data Bank code 1WEK chain F, gray), B. subtilis putative lysine decarboxylase (Protein Data Bank code 1T35 chain B, light pink), and A. thaliana lysine decarboxylase-like protein from gene AT2G37210 (Protein Data Bank code 2A33 chain A, lilac). Figures were generated with MOLSCRIPT (52) and rendered with PovRay.

TABLE 1
Data collection and refinement statistics

| Data set       | Se-Met (MCP1) | Native 1 (MCP2) | Native 2 (MCP3) |
|----------------|---------------|-----------------|-----------------|
| Wavelength (Å) | 0.97962       | 1.005           | 1.5418          |
| Space group    | P21,21,21     | C222,222,222    | C222,222,222    |
| Unit cell dimensions a, b, c (Å) | 68.3, 75.9, 126.6 | 68.1, 126.0, 75.7 | 67.6, 124.5, 74.8 |
| Resolution limits (Å) | 50.0-2.38 | 75.6-1.60 | 62.0-2.32 |
| Completeness (last shell) (%) | 96.5 (88.1) | 95.1 (98.3) | 99.4 (100.0) |
| Rsym (last shell) | 0.064 (0.334) | 0.055 (0.313) | 0.073 (0.364) |
| l/σ(l) (last shell) | 22.6 (2.3) | 35.2 (2.2) | 5.7 (2.1) |

Refinement Statistics

| Observed reflections | 197378 | 182249 | 111712 |
| Unique reflections | 53717 | 38713 | 16374 |
| Residues A: 4–73, 76–166 | A: 3–70, 76–166 | A: 4–73, 76–167 |
| Residues substituted by Ala | A: 72, 73, 76 | A: 72, 73, 76 |
| Water molecules | 162 | 245 | 240 |
| Deviations from ideal values | | | |
| Bond distances (Å) | 0.020 | 0.025 | 0.019 |
| Angle distances (°) | 1.965 | 1.982 | 1.832 |
| Torsion angles (°) | 9.138 | 5.860 | 8.737 |
| Planar groups (Å) | 0.102 | 0.110 | 0.108 |
| Ramachandran statistics | 0.909/0.080/0.012/0.008 | 0.925/0.075/0.008/0.007 | 0.911/0.070/0.019/0.006 |

* Rsym = Σlhkl|Ihkl(I) − (I)|/Σlhkl Ihl(I) where l is the lth measurement and (I) is the weighted mean of all measurements of I.
* (I)/σ(I) indicates the average of the intensity divided by its average standard deviation.
* Rfree, same as Rfree for 5% of the data randomly omitted from the refinement.
* Ramachandran statistics indicate the fraction of residues in the most favored, additionally allowed, generously allowed, and disallowed regions of the Ramachandran diagram, as defined by PROCHECK (42).
**Structure of the Molybdenum Cofactor Carrier Protein**

MCP2 data sets were obtained from apo-MCP, the MCP3 crystal was obtained from Wco-saturated MCP, but no difference density indicated any bound Wco. The same was found for Moco-bound MCP (data not shown). Soaking of MCP crystals with ligand was impossible due to the lability of free Moco, which cannot be chemically synthesized.

**Structural Homologies**—Structure-based homology searches by DALI (44) or EBI-SSM (45) revealed highest homologies to proteins with unknown function. All of these proteins have in common that they are annotated in the Pfam data base as lysine decarboxylase-like proteins (46) due to their homology to the Rossmann fold of lysine decarboxylases (47). Consequently MCP as well as lysine decarboxylase-like proteins show homologies to Rossmann fold-containing proteins on the primary (20) and structural levels. In total, five different structures of lysine decarboxylase-like proteins are available in the data base, three of bacterial origin and two of plant origin. Superposition of MCP with Protein Data Bank codes 2A33 (*Arabidopsis thaliana*), 1WEK (*Thermus thermophilus*), and 1T35 (*Bacillus subtilis*) demonstrate high structural conservation as documented by r.m.s.d.s ranging from 1.5 to 1.9 Å for 125–136 residues (Fig. 3D).

**Characterization of the Moco-binding Site**—Despite the lack of a complex structure, different lines of evidence allowed us to propose a Moco-binding site in MCP. Structural comparison of eight monomers derived from the three MCP structures revealed regions of remarkable structural variations in all MCP monomers, pointing to high mobility (Fig. 4A). Residues with highest deviations (13–15, 45–47, 90–95) cluster in a distinct surface patch (Fig. 4B, highlighted in red). Consistent with these variations, the B-factors of these side-chain residues were 1.5–1.8-fold above the average B-factor of the respective MCP structures. In addition, disordered residues that were replaced by alanines or completely absent in the structures (Table 1 and Fig. 4A, –70–75) are also in close proximity to this surface patch (Fig. 4B, Gly-70 and Ile-76 border the delocalized area). Surface presentation of the MCP tetramer shows that this surface patch of mobile residues forms a cavity (Fig. 4B), which is well suited to bind Moco regarding shape and geometrical restraints as well as electrostatic properties (Fig. 4, B and C).

The sequence alignment of MCP homologues (Fig. 4D) revealed that many conserved and invariant residues are localized within or in close proximity to the above described surface patch. Therefore, we selected 2 of these residues, Met-50 and Pro-69, which are invariant and localized at opposite ends of this depression, to replace them by alanines.

**FIGURE 4. Substrate-binding site of MCP.** A, overview of r.m.s.d. of all monomers of the three crystal structures of MCP (MCP1, chains A–D; MCP2, chains E and F; MCP3, chains G and H). Red indicates missing residues; black indicates r.m.s.d. of 3.0–5.9 Å; and deviations between 0 and 3.0 Å are depicted in shades of gray. The figure was generated using LSQMAN. mRMSD, mean r.m.s.d. of all analyzed chains. B, transparent surface presentation of the MCP tetramer shows that this surface patch of mobile residues forms a cavity (Fig. 4B), which is well suited to bind Moco regarding shape and geometrical restraints as well as electrostatic properties (Fig. 4, B and C).

GRASP (53), and figures were prepared as for Fig. 3. D, sequence alignment of MCP residues 38–75 with homologous proteins from *Crocosphaera watsonii* (EAM49636), *Trichodesmium erythraeum* (EAO26031), *Methanopyrus kandleri* (NP 614673), *Aquifex aeolicus* (NP 213091), *Thermotoga maritima* (NP 228861), *Archaeoglobus fulgidus* (AA890115), *Rhodobacter sphaeroides* (EAP67917), *Synechococcus sp.* (YP 381580), *Dictyostelium discoideum* (EAL69951), *Oryza sativa* (BAD46468), and *A. thaliana* (AA646859). The alignment was generated with ClustalW, and the first residue of each sequence line is shown. E and F, stability of MCP wild type and mutant variants M50A and P69A at 4 °C (E) and 22 °C (F). 1000 pmol of wild type and mutant variants were incubated the indicated times, and MPT was determined by HPLC FormA analysis.
Co-purification of the resulting MCP variants M50A and P69A with bound Moco proved that both variants were able to bind Moco with different saturations. P69A showed the same amount of co-purified Moco as wild type MCP, whereas the binding capacity of M50A was reduced to 50%. However, even more pronounced differences were observed in the ability of these variants to protect bound Moco from degradation. Both variants showed dramatically reduced Moco protection at 4 and 22 °C (Fig. 4, E and F). Within a few hours, more than 80% of bound Moco was degraded. At high temperature, after 1 day, almost all of the ligand was decayed. These findings demonstrate that both residues participate in Moco binding and stabilization.

Besides this experimental evidence, we also performed in silico docking studies (data not shown and Fig. 5) using GOLD (34, 35) with each of the monomer structures and Moco taken from Pichia angusta NR structure (Protein Data Bank code 2BII) (15) but removing all terminal molybdenum ligands as the structure of free Moco (not molybdenum enzyme-bound Moco) is unknown. All docking results (top scores) show Moco bound to the aforementioned surface patch. According to the structural variations in each monomer, the orientation of the docked Moco is slightly different.

Final evidence supporting the binding of Moco to this site comes again from structural comparison with the aforementioned lysine decarboxylase-like proteins. In all three structures shown in Fig. 3D, oxyanions were co-crystallized (Fig. 5). One sulfate (Protein Data Bank code 2A33) and two phosphate anions (Protein Data Bank codes 1WEK and 1T35) occupy almost identical positions. The positions of these anions almost perfectly match with the binding site of the docked Moco phosphate. Moreover, in two structures (Protein Data Bank codes 2A33, 1WEK), residues similar to as MCP are disordered, confirming the above mentioned high structural flexibility in close proximity to the active site.

**DISCUSSION**

*C. reinhardtii* MCP binds Moco and Wco with high affinity. This binding is very selective for the molybdenum- or tungsten-substituted form of the cofactor as MPT did not bind to MCP. Therefore, the protein must exhibit specific properties to recognize the metal part of Moco and Wco. The observed protection of Moco and Wco from degradation is consistent with binding the metal part of the cofactor as it is generally assumed that release of the metal atom goes along with fast degradation. This protection of Moco would be a requirement by oxygen-evolving organisms such as *C. reinhardtii* to keep the highly labile Moco active (18).

It is remarkable that depending on the presence of molybdate or tungstate in the culture medium, different forms of the cofactor were co-purified with MCP. In *C. reinhardtii*, only molybdenum-dependent enzymes have been described so far, suggesting that binding of Wco to MCP has no physiological function. The observed decrease of the three absorption bands specific for Moco-loaded MCP is consistent with the observed degradation of Moco over time. Despite this, slight changes in the spectral properties indicate that the molybdenum center was oxidized during aerobic incubation, and the addition of dithionite reverted this process as indicated by a match of the starting and dithionite-reduced spectrum. The observed absorption bands at 470 and 570 nm point to additional molybdenum charge transfer bonds that must be different from those seen in SOs where a cysteine is ligated to molybdenum (see below). The spectral properties of Wco-loaded MCP were different from those of Moco-loaded MCP. Similar spectral changes have been observed for recombinant human sulfite oxidase upon expression and purification from tungstate-containing medium (37).

Competition studies with either tungstate or molybdate show that Moco is not solvent-exposed during transfer from MCP to nit-1 apo NR. When comparing tungstate inhibition of MCP with the one observed during Moco synthesis, one can argue that MCP protects the molybdenum from being replaced by tungsten as in vitro Moco synthesis is already inhibited with 10 μM tungstate (48). The opposite experiment with Wco-loaded MCP and molybdate as competitor clearly demonstrates no replacement of tungsten, which is consistent with tighter binding of Wco to MCP.

We also investigated the rates of Moco transfer to apo-NR and observed biphasic kinetics, which might be explained by allosteric release and/or transfer of Moco to apo-NR. Due to the tetrameric arrangement of MCP, it might be possible that one Moco molecule is released much faster than the remaining three molecules as indicated by the ratio of reconstituted NR after 2 and 10 h. Comparing the initial transfer phase of MCP (up to 1 h) with the reconstitution of apo-sulfite oxidase by in vitro synthesized Moco (49) shows an ~6-fold lower rate of reconstitution (60 versus 10 min). In light of the different concentrations used in both systems (10 μM Moco versus 4 nM MCP), the transfer of Moco to apo-NR can be considered as fast and efficient. When also taking into account that Moco
binds with high affinity to MCP (due to its efficient co-purification), the observed transfer rates would nevertheless argue for a direct insertion of Moco from MCP into apo-NR via protein-protein interactions as indicated by previous studies (13) This conclusion is further supported by our inhibition studies with tungstate. Due to the high affinity binding of Moco to MCP, one can conclude that binding and storage of Moco is one of the crucial functions of MCP in *Chlamydomonas* metabolism. The fact that the amount of Moco bound to MCP is significantly higher than that of Moco bound to NR confirms such storage function (50).

The structure of MCP shows a symmetric homotetramer with the monomers arranged in a Rossmann-like fold. A distinct surface depression is formed in each monomer, which is accessible within the tetramer and well suited in terms of shape and surface charge to bind Moco. Structure-guided mutagenesis coupled to Moco binding and stability studies confirmed this depression as a potential Moco-binding site. Independent docking studies also point to this region as a potential ligand-binding site. The strongest evidence that the resulting docking model is functionally relevant comes from the match of the Moco-binding site with co-crystallized oxanions. In three different structures, these anions occupy the same position as the phosphate of the docked Moco model. On this basis, one can conclude that the phosphate of Moco is crucial for substrate binding.

It is well known that substrate-binding sites often show high structural flexibility, which is constrained upon ligand binding. For MCP and also for homologous structures, such mobile residues might be the reason for high affinity binding of Moco and its protection against degradation due to its interaction with molybdenum. This view is supported by the identification of specific absorption bands at 470 and 570 nm. Interestingly, *C. reinhardtii* MCP lacks cysteine (20), and so Moco coordination must differ from SOs. Non-covalent binding as well as coordination by other proteinogenic ligands such as aspartate or serine (51) is possible.

Future experiments are needed to finally confirm the proposed Moco-binding site in MCP. Besides knowing the Moco-binding site in MCP, it is even more important to gain information about the atomic structure of Moco before its insertion into a molybdenum enzyme. In eukaryotes, so far Moco was seen in its enzyme-bound forms either ligated to a protein-derived cysteine or ligated with a terminal sulfido ligand. In the literature, free Moco was depicted with two terminal oxo-ligands (2), but recent studies on Moco synthesis suggest the presence of three terminal oxo/hydroxo ligands (49), one of which might be targeted for either cysteine bond formation or sulfuration.

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Structure of the Molybdenum Cofactor Carrier Protein