The *Chlamydomonas reinhardtii* MoCo carrier protein is multimeric and stabilizes molybdopterin cofactor in a molybdate charged form

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Abstract In *Chlamydomonas reinhardtii*, molybdopterin cofactor (MoCo) able to reconstitute active nitrate reductase (NR) with apoenzyme from the *Neurospora crassa* mutant *nit*-1 was found mostly bound to a carrier protein (CP). This protein is scarce in the algal free extracts and has been purified 520-fold. MoCoCP is a protein of 64 kDa with subunits of 16.5 kDa and an isoelectric point of 4.5. In contrast to free MoCo, MoCo bound to CP was remarkably protected against inactivation under both aerobic conditions and basic pH. MocoCP transferred active MoCo to apoNR in vitro without addition of molybdate, though reconstituted activity was 20% higher in the presence of molybdate. Incubation with tungstate specifically inhibited MoCoCP activity but had no effect on the activity of free MoCo released from milk xanthine oxidase. MoCoCP did not charge molybdate unless in the presence of *N. crassa* extracts. Our data support that MoCoCP stabilizes MoCo in an active form charged with molybdate to provide MoCo to apomolybdoenzymes.

Key words: Molybdenum cofactor; Molybdopterin; Tungstate; *Chlamydomonas reinhardtii*

1. Introduction

Molybdenum cofactor (MoCo) is present in all molybdoenzymes, excepting dinitrogenase, with a similar structure. MoCo is a key in oxidoreductases acting on C, N or S atoms, and catalyzing either oxidative hydroxylations or reductive dehydroxylations [1–3]. Elucidation of MoCo structure has been hampered in part because of its easy oxidation by oxygen [4]. MoCo is an alkylated not fully aromatic pterin which occurs in several dihydro forms, depending on the enzyme. The alkylic tetracarbon side chain of MoCo contains a dithiolene group with sulfurs forming a complex with molybdenum [2,3]. In prokaryotes, the cofactor can be modified by an additional nucleotide monophosphate bound to the C4′ phosphate group within the alkylic side chain of the pterin [3,5].

MoCo can be measured by the so-called nit-1 assay which uses the *nit*-1 mutant from *Neurospora crassa* lacking functional MoCo [6]. Addition of MoCo from different sources to extracts of this mutant results in the reconstitution of nitrate reductase (NR) activity from inactive apoNR. Heat (80°C, 90s) or acid treatment is needed to release MoCo from molybdoenzymes [7,8]. Only low M_r free MoCo and MoCo bound to carrier protein (CP) can directly complement in the nit-1 assay without the releasing treatment [2,9]. cDNAs and genes for MoCo biosynthesis have already been cloned in plants and most of the pathway has been dissected [3,10]. However, information on properties of functional MoCo and its insertion into apomolybdoenzymes is still scarce.

In *Chlamydomonas reinhardtii*, MoCo able to directly reconstitute NR in the nit-1 assay is mostly bound to a 50 kDa protein (named molybdenum cofactor carrier protein, MoCoCP) [9]. A similar situation has been described in *Escherichia coli*, where MoCo is loosely bound to a 40 kDa carrier protein which easily dissociates MoCo [11]. MoCoCP from *C. reinhardtii* cannot complement apoNR from *N. crassa nit*-1 mutant through dialysis membranes permeable to free MoCo, which indicates that direct contact between both proteins is needed for complementation [12]. A 70 kDa MoCoCP with characteristics similar to that from *C. reinhardtii* has also been described in *Vicia faba* [13].

C. reinhardtii MoCoCP is a constitutive protein whose abundance depends on the nitrogen source and the growth phase. Transfer of ammonium-grown cells to media containing nitrate, urea or no nitrogen increases MoCo activity up to three times. This process is independent of de novo protein synthesis, requires light and also takes place in NR mutants defective in structural or regulatory genes [9].

In this work, the *C. reinhardtii* MoCoCP has been extensively purified and its properties analyzed. MoCoCP is a multimeric protein which efficiently protects MoCo from inactivation by oxygen. MoCoCP contains MoCo mostly loaded with molybdenum which, in contrast to free MoCo, can be inactivated by tungstate.

2. Materials and methods

2.1. Cells and growth conditions

C. reinhardtii wild type and mutant strain 305 deficient in the structural gene for nitrate reductase have been characterized elsewhere [14]. Cells were grown at 25°C under continuous illumination in liquid minimal medium containing 4 mM KNO_3 as a nitrogen source, and bubbled with 2–5% (v/v) CO_2-enriched air [15]. *Neurospora crassa nit*-1 mutant was grown in ammonium containing medium and induced in 8 mM nitrate, under previously reported conditions [16].

2.2. Preparation of extracts and purification of MoCoCP

Mutant 305 was routinely used as the source of MoCoCP for purification. Cells (about 50 g) were collected from liquid medium by centrifugation at $10\,000 \times g$ for 5 min, and disrupted by freezing at −80°C and thawing in 50 ml distilled water, previously degassed and

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Abbreviations: CP, carrier protein; MoCo, molybdenum cofactor; MPT, molybdopterin; NR, nitrate reductase

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saturated with nitrogen, containing 1 mM DTT, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride, with continuous stirring under nitrogen atmosphere. The suspension was centrifuged at 48 000×*g* for 30 min, and the supernatant used as a crude extract. Ampholyne (1.5 ml) from Pharmacia (range 5–8) was added to the above solution. After mixing, the solution was charged to the Rotophor cell from Bio-Rad. Isoelectric focusing was run at 12 W constant power. After 5 h, fractions were collected and MoCoCP activity determined in 1 μl samples. Fractions with activity were pooled. Distilled water saturated with N_2 and containing 1 mM DTT was added up to a final volume of 55 ml. A second isoelectric focusing was run as above. Samples with MoCoCP activity were collected and fractions with MoCoCP activity were pooled and concentrated by ultrafiltration in Centricon filter (30 kDa cut off) to a final volume of 2.5 ml.

The sample, to which glycerol and the tracking dye were added, was loaded on a 491 PrepCell from Bio-Rad. Preparative electrophoresis was carried out at 4°C on 10% polyacrylamide gels in thoroughly degassed 50 mM Tris acetate buffer, pH 8.0, containing 1 mM DTT, 1 mM EDTA, at 12 W constant power. Gels of 8.0 cm high were previously subject to pre-electrophoresis for 30 min with 900 μM thioglycolic acid in the cathodic chamber and in the elution buffer. Elution was performed at a flow rate of 0.75 ml/min and fraction collection (4.9 ml/fraction) started after 10 h 45 min of electrophoresis. The elution buffer was continuously bubbled with N_2. Fractions with MoCoCP activity were pooled and concentrated to 1.5 ml through a 10 kDa ultrafiltration membrane under N_2 atmosphere in a Millipore chamber. Purified MoCoCP was stored in a 50 mM Tris acetate buffer, pH 8.0, containing 1 mM DTT, 1 mM EDTA, degassed and saturated with N_2 (buffer M) at −80°C.

2.3. Electrophoresis and isoelectric focusing

SDS-PAGE was performed in porous gels as detailed in the text and according to Laemmli [17] and Doucet and Trifaró [18]. Molecular weight markers used in SDS gels were from SIGMA. Gels were silver-stained at 37°C according to the method of Nielsen and Brown [19].

Polyacrylamide isoelectric focusing gels (PhastGel, IEF 3–9, Pharmacia LKB) were subject to isoelectric focusing under native conditions in a PhastSystem apparatus following the manufacturer's instructions. The isoelectric point of purified proteins was obtained by comparing in silver-stained gels the migration of proteins with IEF markers from Pharmacia.

2.4. Molecular exclusion chromatography

Size fractionation of proteins (in 100 μl samples) was performed by FPLC in a Superdex 200 column (1×30 cm) in 50 mM Tris acetate buffer, pH 8.0, containing 1 mM DTT, and 1 mM EDTA, and saturated with nitrogen. The chromatography was run at a flow rate of 0.5 ml/min and fractions of 0.33 ml were collected. Human carbonic anhydrase, 29 kDa, and bovine serum albumin, 66 kDa, were used as M_r markers.

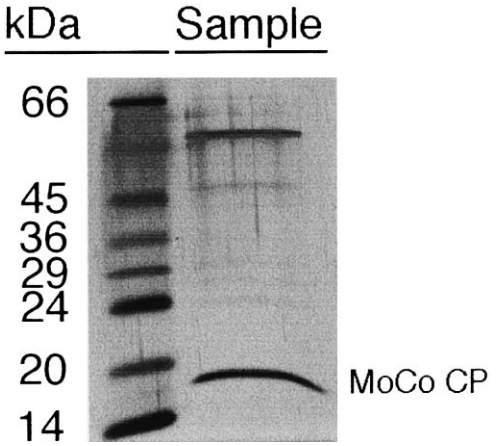

Fig. 1. SDS-PAGE of purified MoCoCP from *C. reinhardtii*. A sample (0.65 μg) of purified MoCoCP was analyzed by SDS-PAGE and silver-stained. M_r markers are indicated.

2.5. MoCo and MoCoCP activity

Active MoCo was assayed by determining the reconstituted NR activity as previously described [6] without any treatment for MoCo release [12]. The *N. crassa nit*-1 mutant extracts were obtained in 50 mM phosphate buffer, pH 7.2, containing 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF, and 100 mM NaCl, and distributed in aliquots which were frozen at −80°C until use. Routinely 0.5 μl of MoCo-containing preparations were incubated with 50 μl of the *N. crassa* extract for 1 h at 15°C. Then, NADPH-NR activity was assayed as reported elsewhere [20]. One unit of MoCo is defined as the amount of MoCo that yields one unit of reconstituted NR activity, defined as the amount of enzyme that catalyzes the reduction of 1 μmol of nitrate per min. Free MoCo was extracted from milk xanthine oxidase by heat treatment (80°C, 90 s) of enzyme preparations 50-fold diluted in buffer M [8].

2.6. Analytical methods

Nitrite was determined according to Snell and Snell [21]. Protein was determined using previously reported methods [22,23]. Chemical determination of MoCo was carried out by analyzing molybdopterin (MPT) converted into dephospho form A as previously described [24].

3. Results

3.1. Purification of MoCoCP

The MoCoCP in *C. reinhardtii* was able to complement efficiently apoNR from the *N. crassa nit*-1 mutant in direct reconstitution assays, which did not include molybdate or MoCo release treatments [12]. This reconstitution by MoCoCP only took place with the excluded fraction (from a Sephadex G-25) of the *N. crassa nit*-1 mutant extracts, which

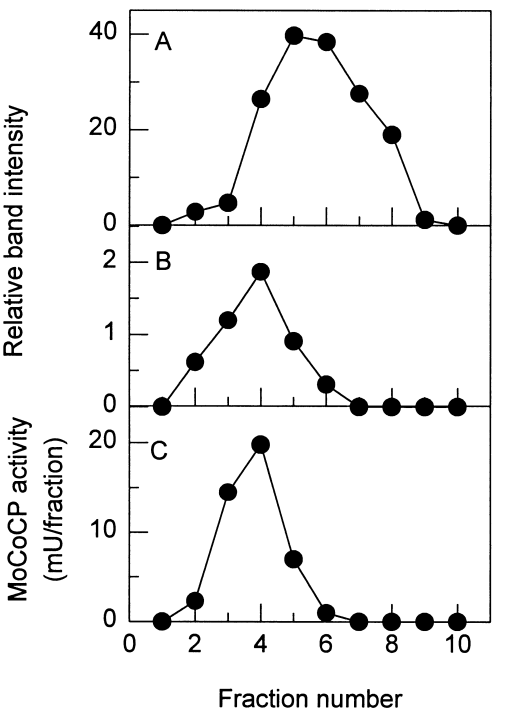

Fig. 2. Elution profile of purified MoCoCP preparations after gel exclusion chromatography. Purified MoCoCP was subjected to chromatography through a Superdex 200 column as described in Section 2. After eluting 11.67 ml, 10 fractions were collected in which MoCoCP activity was determined (C). A sample from each was analyzed by SDS-PAGE and the amounts of the 54 kDa (A) and 16.5 kDa (B) proteins were determined in arbitrary units from the densitometric analysis of the corresponding bands in silver-stained SDS gels.

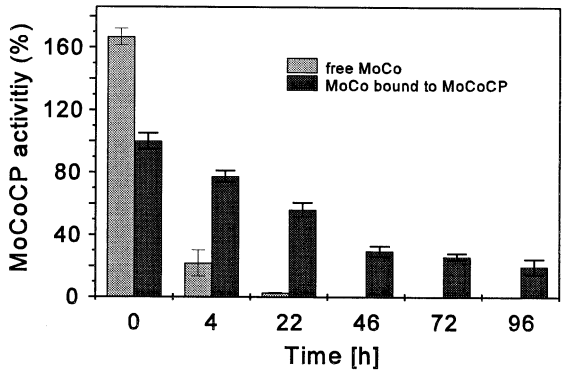

Fig. 3. Inactivation of MoCoCP from *C. reinhardtii* and free MoCo from milk xanthine oxidase under air atmosphere. Purified MoCoCP and free MoCo from xanthine oxidase were incubated in aerobic conditions, and samples were taken at the indicated times and MoCo activity determined. The experiment was carried out in triplicate and mean values and standard deviations are shown for each time. 100% MoCo activity corresponded to 0.47 U/ml.

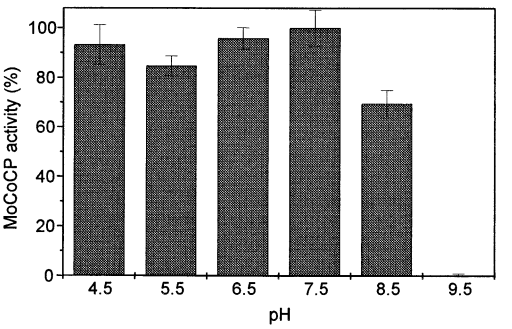

Fig. 4. Effect of pH on MoCoCP activity. Purified MoCoCP was incubated under nitrogen atmosphere for 5 h at 4°C in buffers at a concentration of 50 mM of acetate, pyridine, MES, MOPS, Tris, and Bis-Tris-propane and the indicated pHs ranging from 4.5 to 9.5 containing 1 mM DTT. 100% MoCo activity corresponded to 1.1 U/ml. Other conditions as in Fig. 2.

rules out that MoCoCP is converting precursors from *N. crassa* into functional MoCo (results not shown).

Purification of MoCoCP was carried out by following MoCo activity. Since MoCo was readily inactivated in aerobic conditions, all buffers were degassed and flushed with N_2. In addition, pH was found to be a key factor to preserve MoCoCP activity during chromatography or electrophoresis procedures, so it was routinely maintained at values below 8.0 (see below). MoCoCP has been purified 520-fold with a recovery of about 4% of active MoCo by following the protocol described in Section 2 (Table 1). The purified protein was not homogeneous. Two major protein bands could be detected in silver-stained gels subject to SDS-PAGE (Fig. 1). MoCoCP is assigned to the 16.5 kDa protein, and not to the 54 kDa protein or any other minor one, since the elution profile of MoCoCP activity from purified preparations upon gel exclusion chromatography in Superdex 200 only coincided with the amount of the 16.5 kDa protein detected in silver-stained SDS gels from the eluted fractions (Fig. 2). Special care should be taken during protein staining of SDS gels since the longer the incubation times the lower the amounts of the 16.5 kDa protein detected due to its release from the gels. Even so, the relative abundance of the 54 kDa protein was variable from one purification to another. The molecular size of MoCoCP in native form deduced from gel exclusion chromatography is 64 kDa, and thus MoCoCP corresponds to a protein with four subunits of identical size. A concentration of 0.86 μmol of MPT (dephospho form A) per μmol of protein was found in purified MoCoCP preparations, assuming that the protein measured was homogeneous. Since this is not the case, the molar ratio of MPT in MoCoCP would be higher. Isoelectric focusing of the purified protein indicated that both MoCoCP and the 54 kDa protein had an isoelectric point close to 4.5 (results not shown). These purified preparations were used for a further functional characterization of MoCoCP.

3.2. Functional properties of MoCoCP

MoCo was stabilized against inactivation under aerobic conditions when bound to the CP. MoCo released from milk xanthine oxidase by heat treatment had only about 10% of the initial MoCo activity after 4 h incubation in aerobic conditions, whereas MoCo bound to CP was still more than 80% active (Fig. 3). A half-life of about 24 h has been estimated for MoCo when bound to CP, which is more than one order of magnitude higher than that for MoCo in free form.

MoCo bound to CP was stable (85–100% activity) under nitrogen atmosphere when incubated for 5 h at 4°C with 1 mM DTT in different buffers at a concentration of 50 mM (acetate, pyridine, MES, MOPS) and pHs ranging from 4.5 to 7.5. However, at pH 8.5 (Tris buffer) MoCoCP activity was slowly inactivated so that after 5 h incubation it still retained 69% of the initial activity, and at pH 9.5 (Bis-Tris-propane buffer) no activity could be detected (Fig. 4). This pH effect contrasts with the reported instability of free MoCo which readily loses molybdate above pH 7.6 [25].

The effect of different concentrations of tungstate on MoCoCP activity has been examined (Fig. 5). Incubation of MoCoCP in the standard buffer M with 5 mM tungstate resulted in the inactivation of MoCo with a half-life of 7 h (Fig. 5B). Tungstate (50 mM)-inactivated MoCo bound to CP very quickly since its half-life under these conditions was only 10 min (Fig. 5A). This inhibition took place directly during the incubation of MoCoCP with tungstate without the participation of the *N. crassa nit*-1 extract, since tungstate had no effect during the complementation assay of MoCoCP at the concen-

Table 1
Purification of MoCoCP

Step	Volume (ml)	Protein (μg/ml)	Activity (U/ml)	Specific activity (U/mg protein)	Recovery (%)	Purification (fold)
1. Crude	56	4680	0.77	0.164	100	1
2. Rotophor	12	473	0.53	1.13	15	7
3. Preparative PAGE	1.5	13	1.11	85.38	3.9	522

For details see Section 2.

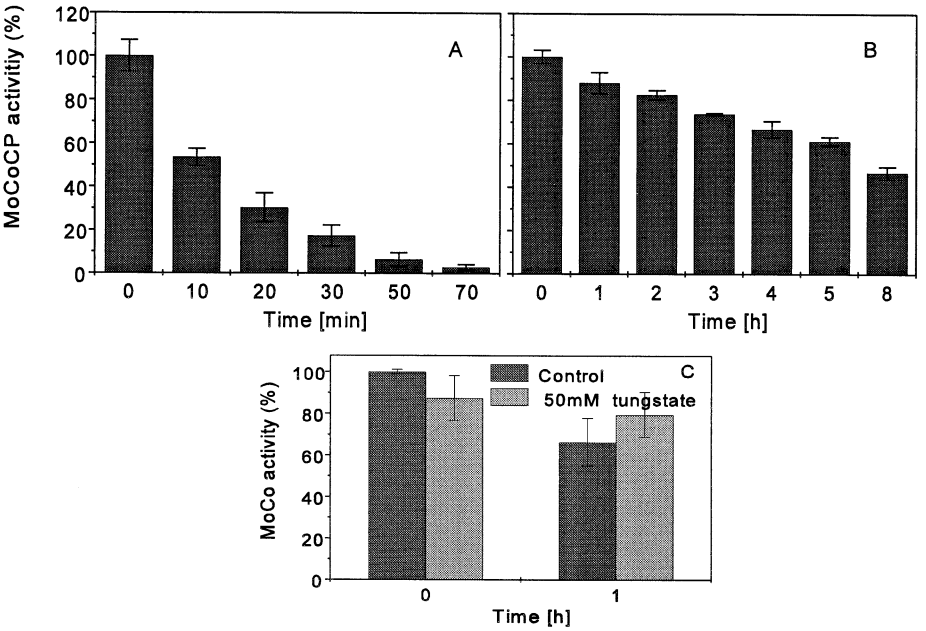

Fig. 5. Inactivation by tungstate of MoCoCP from *C. reinhardtii* and free MoCo from milk xanthine oxidase. Samples of 3.6 μl of purified MoCoCP were incubated under aerobic conditions with 0.4 μl of 10-fold concentrated tungstate solution to give a final concentration of 50 mM (A) and 5 mM (B) in buffer M. Free MoCo from xanthine oxidase was incubated with 50 mM tungstate in the same buffer (C). 100% MoCoCP and free MoCo correspond to 1.5 U/ml and 0.48 U/ml, respectively. The ratio volumes of MoCo:nit1 extracts was 0.5 μl:50 μl in the nit-1 assay, so that the final concentrations of tungstate were 0.05 and 0.5 mM. Controls to determine the effect of tungstate at these concentrations on the nit-1 assay were performed and rendered 119 ± 14% and 131 ± 15%, respectively. Other details as in Fig. 2.

trations corresponding to those in the experiments of Fig. 5A,B. In contrast to MoCo bound to CP, free MoCo released from milk xanthine oxidase was not inhibited by tungstate. As shown in Fig. 5C, 50 mM tungstate did not cause inhibition of free MoCo activity after 1 h incubation when compared with control values. In fact, inhibition of free MoCo under air (control) was smaller in the presence of tungstate (Fig. 5C). These results suggest that MoCo loaded with molybdate and bound to CP is able to exchange molybdate by tungstate, converting MoCo into an inactive cofactor.

MoCoCP activity was 20% higher when assays were performed in the presence of 5 mM molybdate, which suggests that MoCoCP contains a fraction of demolybdo cofactor. To load with molybdate this demolybdo MoCo, MoCoCP was incubated anaerobically in buffer M with increasing concentrations of molybdate from 5 μM to 5 mM during 15 h. However, no increase in MoCoCP activity over the control value (no molybdate added) was found, unless 5 mM molybdate was included during the *N. crassa nit*-1 mutant reconstitution assay.

4. Discussion

The molybdopterin cofactor carrier protein has been purified 550-fold from *C. reinhardtii* extracts to obtain further insight into its properties. Two factors, pH below 8.0 and anaerobiosis, are important to stabilize MoCo bound to CP long enough to apply purification techniques to MoCoCP isolation. Even so, the low abundance of MoCoCP only allowed us to recover small amounts of purified protein. MoCoCP showed a molecular size of 64 kDa and an isoelectric point of 4.5 in native form and corresponds to a protein with subunits of 16.5 kDa, which strongly suggests that MoCoCP is a homotetramer. The size of the *C. reinhardtii* MoCoCP is consistent with that reported previously by Aguilar et al. [9] for this protein. In *Vicia faba*, MoCoCP was also purified but to an extremely low specific activity. The protein was reported to be a 70 kDa single polypeptide chain [13]. Considering the specific activity of 25 U/ng-atom Mo reported for MoCo in the reconstituted *N. crassa* NR assay for 24 h at 3.5°C [26], the specific activity of MoCo in our purified preparation (85.4 U/mg protein) is high since it corresponds to about 5.55 U/ng-atom Mo (assuming 1 MoCo per molecule) in our standard reconstitution assay (1 h at 15°C), which might have underestimated the actual MoCo amounts [26,27].

The properties of MoCo bound to CP are distinct from those of free MoCo. As is well known, free MoCo is very unstable under aerobic conditions and shows half-lives of around 1 h depending on the components in the buffer solution [26,27]. Notwithstanding, MoCo in CP was stabilized against air inactivation and showed a half-life of 24 h, long enough to ensure an efficient storage of active MoCo. During the oxygenic light phase of plants, stabilization of synthesized MoCo against oxygen might be a critical step for its efficient transfer to apomolybdoenzymes. MoCo in CP was also stabilized against pHs below 8.5, whereas free MoCo readily loses molybdate [25] and is inactivated above pH 7.6, which indicates that MoCoCP stabilizes the MoCo pyrano ring against basic pH. These results suggest that MoCoCP binds MoCo so that the free access of media constituents to the MPT structure is prevented.

Moreover, MoCoCP activity was also readily inactivated by tungstate, in contrast to free MoCo, which strongly suggests that molybdate can be exchanged in the MoCoCP by tungstate, thus causing inactivation of Moco. The efficiency of this inactivation was dependent on tungstate concentration, so

that at 50 mM tungstate MoCo activity of CP was significantly inactivated within minutes. Another important fact is that 5 mM molybdate included in the apoNR reconstitution assay only slightly activated MoCoCP activity (about 20%), whereas free Moco was strongly activated (about three times) by this concentration of molybdate, as previously reported [8,26]. This different degree of activation might reflect that most MoCo in CP is already charged with molybdate, but free MoCo is present mainly in a demolybdo form as previously proposed [26,27]. That incubation of MoCoCP with molybdate did not activate bound MPT unless molybdate was also present in the *N. crassa nit*-1 extracts suggests that the molybdate charging of MoCo in CP is mediated by some enzyme present in the fungal extract.

These clear differences between the behavior of free Moco and MoCo bound to CP against tungstate and molybdate indicate that MoCoCP plays an important role in maintaining MoCo in active form both by protecting MoCo from oxygen inactivation and by keeping MoCo in a molybdate-charged form.

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References

[1] Coughlan, M.P. (1980) in: Molybdenum and Molybdenum-containing Enzymes (Coughlan, M.P., Ed.), Pergamon Press, Oxford.
[2] Rajagopalan, K.V. and Johnson, J.L. (1992) J. Biol. Chem. 267, 10199–10202.
[3] Mendel, R.R. (1997) Planta 203, 399–405.
[4] Johnson, J.L. (1980) in: Molybdenum and Molybdenum-containing Enzymes (Coughlan, M.P., Ed.), pp. 345–383, Pergamon Press, Oxford.
[5] Krüger, B. and Meyer, O. (1988) Biochim. Biophys. Acta 912, 357–364.
[6] Nason, A., Lee, K.Y., Pan, S.S., Ketchum, P.A., Lamberti, A. and De Vries, J. (1971) Proc. Natl. Acad. Sci. USA 68, 3242–3246.
[7] Mendel, R.R. (1983) Phytochemistry 22, 817–819.
[8] Mendel, R.R., Alikulov, Z.A. and Müller, A.J. (1982) Plant Sci. Lett. 25, 67–72.
[9] Aguilar, M., Cárdenas, J. and Fernández, E. (1990) Biochim. Biophys. Acta 1073, 463.
[10] Stallmeyer, B., Nerlich, A., Schiemann, J., Brinkmann, P. and Mendel, R.R. (1995) Plant J. 8, 751–762.
[11] Amy, N.K. and Rajagopalan, K.V. (1979) J. Bacteriol. 140, 114–124.
[12] Aguilar, M., Kalakoutskii, K., Cárdenas, J. and Fernández, E. (1992) FEBS Lett. 307, 162.
[13] Kalakoutskii, K. and Fernández, E. (1996) Planta 201, 64–70.
[14] Fernández, E. and Cárdenas, J. (1982) Mol. Gen. Genet. 186, 164–169.
[15] Harris, E. (1989) The Chlamydomonas Sourcebook, Academic Press, New York.
[16] Garrett, R.H. (1972) Biochim. Biophys. Acta 264, 481–489.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Doucet, J.P. and Trifaró, J.M. (1987) Anal. Biochem. 168, 265–271.
[19] Nielsen, B. and Brown, L. (1984) Anal. Biochem. 141, 311–315.
[20] Paneque, A. and Losada, M. (1966) Biochim. Biophys. Acta 128, 202–204.
[21] Snell, F.D. and Snell, C.T. (1949) Colorimetric Methods of Analysis, Vol. 2, Van Nostrand, New York.
[22] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[23] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[24] Schwarz, G., Boxer, D.H. and Mendel, R.R. (1997) J. Biol. Chem. 272, 26811–26814.
[25] Wahl, R.C., Hageman, R.V. and Rajagopalan, K.V. (1984) Arch. Biochem. Biophys. 230, 264–273.
[26] Hawkes, T.R. and Bray, R.C. (1984) Biochem. J. 219, 481–493.
[27] Rajagopalan, K.V. (1989) in: Molecular and Genetic Aspects of Nitrate Assimilation (Wray, J.L. and Kinghorn, J.R., Eds.), pp. 212–221, Oxford Science Publications, Oxford.