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Direct transfer of molybdopterin cofactor to aponitrate reductase from a carrier protein in *Chlamydomonas reinhardtii*

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A *Chlamydomonas reinhardtii* molybdenum cofactor (MoCo)-carrier protein (CP), capable of reconstituting nitrate reductase activity with apoprotein from the *Neurospora crassa* mutant *nit*-1, was subjected to experiments of diffusion through a dialysis membrane and gel filtration. CP bonded firmly MoCo and did not release it efficiently unless aponitrate reductase was present in the incubation mixture. Stability of MoCo bound to CP against air and heat was very similar to that of free-MoCo released from milk xanthine oxidase. Our data strongly suggest that MoCo is directly transferred from CP to aponitrate reductase to form an active enzyme.

Molybdenum cofactor; Molybdopterin; Nitrate reductase; Xanthine oxidase

1. Introduction

Except for dinitrogenase, all molybdoenzymes studied appear to contain molybdopterin [1–3]. Much is known about the structure of molybdopterin and one of its precursors [4,5] and about genes involved in its biosynthesis [2,6–9] but proteins involved in the pathway from early precursors and molybdate to the final incorporation of MoCo into apoenzymes are poorly understood. Only in a prokaryotic organism, *Escherichia coli* [10], and in a eukaryotic one, *Chlamydomonas reinhardtii* [11], a 40–50 kDa protein, named carrier protein (CP), that binds active MoCo and is not a molybdoenzyme [10,11], has been found. Its function is not clear, however, in *E. coli* the instability of active MoCo has been related with the lability of the binding of MoCo to this CP [10]. The free-MoCo which is also present in extracts from *E. coli* and *C. reinhardtii* may not necessarily exist in vivo, since in vitro it could be released from the carrier proteins with which it is associated [10,11].

In a previous work we proposed that the MoCo-CP from *C. reinhardtii* is able to reconstitute directly NR activity with apoprotein from *N. crassa nit*-1 mutant [11]. Data presented herein strongly support that proposal.

Abbreviations: CP, carrier protein; MoCo, molybdenum cofactor; NR, nitrate reductase; XO, xanthine oxidase.

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2. MATERIALS AND METHODS

Cells of *C. reinhardtii* wild-type 6145c (from Dr. R. Sager, Hunter College, NY) were grown in liquid minimum medium with CO_2-enriched air (5% v/v) containing 10 mM ammonium chloride [12]. Cells were harvested at the mid-exponential phase of growth and stored at −40 °C until use. Mutant strain *nit*-1 of *N. crassa* was grown aerobically in basal Fries medium containing ammonium [13] and aponitrate reductase was induced as described previously [11].

Extracts from *C. reinhardtii* cells were obtained by gently thawing the frozen cell pellets in buffer A (25 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 1 mM PMSF) degassed with O_2-free argon (2 ml/g fresh weight) as detailed elsewhere [11]. Extracts from *nit*-1 mycelia were obtained in buffer B (25 mM potassium phosphate, pH 7.5, containing 25 mM sodium molybdate and 1 mM EDTA) as reported [11].

MoCo was released from milk xanthine oxidase (XO) preparations by heating at 80°C for 90 s [11]. *C. reinhardtii* MoCo bound to CP was routinely obtained by filtration of extracts through a Sephadex G-25 column (1.5×8 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.0, unless otherwise indicated. Active MoCo was assayed by determining the reconstituted nitrate reductase activity as previously described [11] in a mixture of 200 μl of *N. crassa nit*-1 mutant extract and 100 μl of the MoCo source after preincubation under optimum complementation conditions at 15°C during 1–3 h. One unit of active MoCo is defined as the amount of MoCo that yielded one unit of reconstituted NR activity expressed as the amount of enzyme which catalyzes the reduction of 1 μmol of nitrate per min. Complementation experiments through a dialysis membrane were carried out by placing 1.5 ml of extract of *N. crassa nit*-1 mutant in a dialysis bag opened at one side and immersed into the MoCo source. At the indicated times, 100 μl-samples were taken for MoCo assays which were performed during 5 min as indicated above. Nitrite was estimated colorimetrically according to Snell and Snell [14] and protein was measured according to Smith et al. [15] using bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

Extracts from ammonium-grown cells of *C. reinhardtii* show most of their MoCo activity associated

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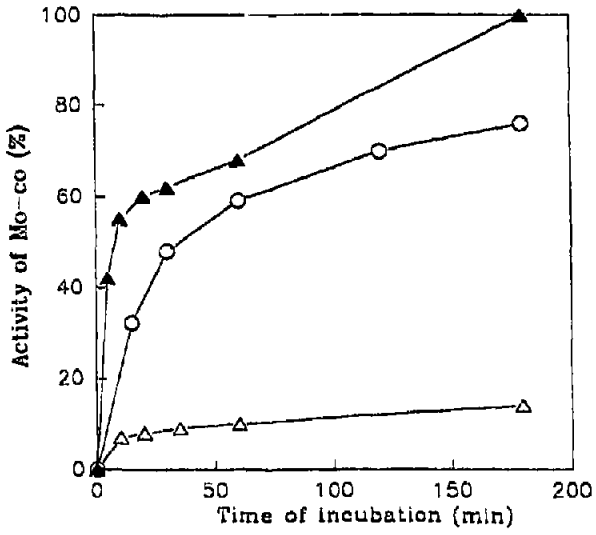

Fig. 1. Kinetics of direct and nondirect complementation between MoCo bound to CP from *C. reinhardtii* or free-MoCo from milk XO and apoNR from *nit*-1 mutant of *N. crassa*. Complementation was carried out at 20°C either directly between crude extracts containing CP-MoCo and apoNR from *nit*-1 (filled triangle) or nondirectly (through a dialysis membrane) between CP-MoCo and apoNR (open triangle), and between free-MoCo from milk XO (released by heat treatment) and apoNR (circle). One hundred percent of MoCo activity corresponded to 8.2 and 5.1 mU for MoCo bound to *C. reinhardtii* CP and MoCo from XO, respectively, in 3-h direct complementation assays. Other experimental details are described in Materials and Methods.

with a carrier protein [11]. When extracts of *C. reinhardtii* containing MoCo bound to CP were incubated at 15°C for 0–75 min and then subjected to gel filtration, more than 90% of MoCo remained bound to CP. To determine whether MoCo bound to CP could be directly transferred to apoNR of *N. crassa nit*-1 mutant without previous dissociation of MoCo from CP, we compared the kinetics of direct and nondirect (through a dialysis membrane) complementation of MoCo from *C. reinhardtii* and apoNR from *N. crassa nit*-1 mutant (Fig. 1). Free-MoCo released from milk XO was used as a control of the nondirect complementation. Unlike free MoCo from milk XO, MoCo bound to CP was not efficiently released and so did not diffuse through the dialysis membrane (Fig. 1). Reconstitution of NR activity by free-MoCo from XO (nondirect complementation) and CP-MoCo (direct complementation) took place to a similar extent and with a similar kinetics. All these results indicate that *C. reinhardtii* MoCo-CP is directly taking part in the transfer of MoCo to apoNR. Since CP cannot pass through the dialysis membrane and binds strongly MoCo, a physical contact between CP and apoNR either direct or mediated by any other protein is required. Unlike CP of *C. reinhardtii*, that of *E. coli* binds MoCo very loosely [10].

MoCo-CP of *E. coli* protects active MoCo from inactivation by heat or oxygen [6,10]. In contrast, in *C. reinhardtii* MoCo associated with CP was highly sensitive to air and showed an inactivation pattern very similar to that of free-MoCo released from milk XO (half-lives about 15 min at 3°C). An increase of temperature up to 15°C resulted in shorter half-lives (about 7 min). These inactivating effects were much protected by the presence of thiol group reductants, specially 1 mM DTT that maintained high the levels of active MoCo bound to CP even after aerobic incubation for 5 h. These results are in agreement with those previously reported for free-MoCo from different sources [17–19] and appear to indicate that in *C. reinhardtii* binding of MoCo to CP does not protect from inactivation.

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