

Molybdoenzymes and molybdenum cofactor in plants

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Abstract

The transition element molybdenum (Mo) is essential for (nearly) all organisms and occurs in more than 40 enzymes catalysing diverse redox reactions, however, only four of them have been found in plants. (1) Nitrate reductase catalyses the key step in inorganic nitrogen assimilation, (2) aldehyde oxidase(s) have been shown to catalyse the last step in the biosynthesis of the phytohormone abscisic acid, (3) xanthine dehydrogenase is involved in purine catabolism and stress reactions, and (4) sulphite oxidase is probably involved in detoxifying excess sulphite. Among Mo-enzymes, the alignment of amino acid sequences permits domains that are well conserved to be defined. With the exception of bacterial nitrogenase, Mo-enzymes share a similar pterin compound at their catalytic sites, the molybdenum cofactor. Mo itself seems to be biologically inactive unless it is complexed by the cofactor. This molybdenum cofactor combines with diverse apoproteins where it is responsible for the correct anchoring and positioning of the Mo-centre within the holo-enzyme so that the Mo-centre can interact with other components of the enzyme's electron transport chain. A model for the three-step biosynthesis of Moco involving the complex interaction of six proteins will be described. A putative Moco-storage protein distributing Moco to the apoproteins of Mo-enzymes will be discussed. After insertion, xanthine dehydrogenase and aldehyde oxidase, but not nitrate reductase and sulphite oxidase, require the addition of a terminal sulphur ligand to their Mo-site, which is catalysed by the sulphur transferase ABA3.

Key words: Abscisic acid biosynthesis, aldehyde oxidase,

molybdenum cofactor, nitrate reductase, sulphite oxidase, xanthine dehydrogenase.

Introduction

It has long been known that the rare transition element molybdenum is an essential micronutrient for plants, animals and microorganisms (Bortels, 1930). However, Mo itself seems to be catalytically inactive in biological systems until it is complexed by a special cofactor. With the exception of the bacterial nitrogenase, Mo is bound to a unique pterin compound named molybdenum cofactor (Moco; Fig. 1A) which binds to diverse apoproteins. These Mo-enzymes participate in essential redox reactions in the global C-, N-, and S-cycles (Hille, 1996). The Moco occurs ubiquitously in all organismic kingdoms. From the more than 40, mostly bacterial, Mo-enzymes (reviewed by Hille, 1996; Sigel and Sigel, 2002), only four have been found in plants. In the first half of this article, the importance of these four Mo-enzymes for the plant will be reviewed, and in the second part the present knowledge about the biosynthesis of Moco in plants will be summarized.

Mo-enzymes in plants

Now that more sequence information is available for Mo-enzymes, the alignment of amino acid sequences permits regions and domains that are well conserved among different Mo-enzymes and other regions that are highly variable in sequence, mostly serving as interdomain hinge regions, to be defined. The atomic structures of chicken SO (Kisker *et al.*, 1997a) and of bovine xanthine oxidase (Enroth *et al.*, 2000) were determined. These first crystal structures of eukaryotic Mo-enzymes combined with the wealth of sequence knowledge were a great leap forward

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Abbreviations: ABA, abscisic acid; AO, aldehyde oxidase; MPT, molybdopterin; Mo, molybdenum; Moco, molybdenum cofactor; NR, nitrate reductase; SO, sulphite oxidase; XDH, xanthine dehydrogenase.

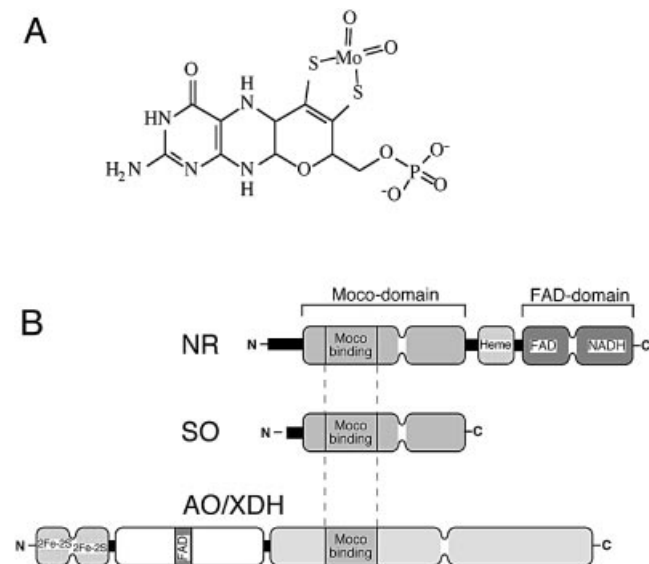


Fig. 1. (A) Structure of Moco. The unique pterin compound molybdopterin co-ordinates Mo via its dithiolene group. (B) Domain arrangement of plant Mo-enzymes. In the active state, all enzymes are homodimeric, here only the monomers are shown. The domains are linked by hinge regions (drawn in black). Constrictions within the domains indicate subdomains. To this end, only the crystal structure for the haem and FAD-domain of NR has been solved (Campbell, 1999), however, on the basis of sequence analyses and the existing atomic structures for animal SO (Kisker *et al.*, 1997a) and xanthine oxidase (Enroth *et al.*, 2000), the subdomain arrangement shown is also likely to occur in the plant Mo-enzymes.

for Mo-enzyme research. It is remarkable that the four Mo-enzymes described in plants are homodimeric proteins functioning only as a dimer, not as a monomer. They are involved in redox processes and (with the exception of SO) harbour an electron transport chain involving different prosthetic groups (FAD, haem or Fe-S, Moco) that are bound to separate domains identified on the enzyme's monomer. Figure 1B shows that the depicted Mo-enzymes fall into two classes sharing similar domains: one group is formed by NR and SO, and another group by XDH and AO. Eukaryotic AO and XDH have very similar amino acid sequences so that it is suggested that they evolved from a common ancestor (Sigel and Sigel, 2002). Nothing is known about how and when FAD and haem become bound to the proteins. For Moco, however, the first crystallographic analyses of Mo-enzymes demonstrated that the cofactor is deeply buried within the holo-enzyme so that Moco could only have been incorporated prior to or during completion of folding and dimerization of the apoprotein monomers (Kisker *et al.*, 1997b; Enroth, *et al.*, 2000).

Nitrate reductase

NR (EC 1.6.6.1) is a cytoplasmic enzyme and has a molecular mass of about 200 kDa for the dimer. There are recent reviews about different aspects of NR research (see below) so that only some points will be given here. As can

be seen from Fig. 1B, the monomer of plant NR consists of three functional domains: the N-terminal domain associated with Moco, the central haem domain, and the C-terminal FAD-domain, each redox-active prosthetic group is bound to the monomer in a ratio of 1:1:1 (reviewed by Campbell, 1999). These domains are connected by protease-sensitive hinge regions. The three domains form three redox centres catalysing the transfer of electrons from the reductant NAD(P)H via FAD, haem, and Moco to nitrate. Both the FAD-domain and the Moco-domain are structurally characterized by subdomains (Campbell, 1999; Kisker *et al.*, 1998).

NR catalyses the first step in nitrate assimilation, a pathway that is of key importance for plant nutrition. Regulation of nitrate assimilation is part of a complex regulatory network responding to diverse environmental and internal signals such as nitrate, light, CO₂, phytohormones, and metabolites of carbon and nitrogen metabolism in order to co-ordinate nitrate assimilation with other key metabolic processes (for review see Crawford and Forde, 2002; Campbell, 1999). The regulation of NR involves both transcriptional and post-translational mechanisms regulating the amount as well as the activity of NR protein. Of special interest is the regulative phosphorylation of the NR protein. In the dark, the NR protein is phosphorylated thereby allowing the stoichiometric binding of a NR inhibitor protein which belongs to the class of 14-3-3 proteins (for review see Kaiser and Huber, 2001). On illumination of leaves, NR is rapidly reactivated by dissociation of the inhibitor protein and dephosphorylation. The inhibitor protein cannot inhibit dephosphorylated NR.

The signal transduction cascade starting at the nitrate transporters (Forde, 2000) and linking availability of nitrate to the induction of transcription is still enigmatic, but receives a lot of attention in research nowadays. There are indications that nitrate serves not only as a substrate for assimilation, but also as a regulatory signal for co-ordinating nitrogen and carbon metabolism (Forde, 2002; Hänsch *et al.*, 2001; Scheible *et al.*, 1997) and driving root development (Zhang and Forde, 2000).

Aldehyde oxidase

AO (EC 1.2.3.1) is a cytoplasmic enzyme with an apparent molecular mass of 300 kDa, containing FAD, iron and Moco as prosthetic groups (Koshiba *et al.*, 1996). The domain arrangement of AO (Fig. 1B) shows that the redox-active iron is incorporated in the form of Fe-S centres that are localized on the N-terminal part. The protein monomer of the homodimeric enzyme binds Fe-S, FAD, and Moco in a stoichiometric ratio of 4:1:1. The AO gene was cloned from maize (Sekimoto *et al.*, 1997), tomato (Ori *et al.*, 1997) and *Arabidopsis* where four AO cDNAs were found and physically mapped to different chromosomes (Sekimoto *et al.*, 1998). The encoded enzyme isoforms

have a relatively broad substrate specificity for several aldehydes including abscisic aldehyde, indole-3-aldehyde, indole-3-acetaldehyde, and benzaldehyde. From the substrate specificity, mutant analysis and tissue distribution it can be concluded that *Arabidopsis* AO3 catalyses the conversion of abscisic aldehyde to abscisic acid (ABA) (Seo *et al.*, 2000), the final step in ABA-biosynthesis (for details see the recent reviews of Seo and Koshiba, 2002; Milborrow, 2001; Zeevaert, 1999). A different line of experimental approaches linked AO activity to the biosynthesis of the plant hormone indole-3-acetic acid (IAA), where it catalyses the conversion of indole-3-acetaldehyde to IAA: AO1 activity is five times higher in the IAA-overproducing mutant *sur1* as compared to the *Arabidopsis* wild type (Seo *et al.*, 1998).

Obviously plant AOs form a multigene family whose members catalyse the final step in the biosynthesis of the phytohormones ABA and probably IAA. These two functions are sufficient to assign an important role in plant development and adaptation to environmental stresses to AOs although there are also shunt pathways for the synthesis of both hormones (Milborrow, 2001; Slovin *et al.*, 1999). The broad substrate specificity of AO makes it likely that AOs are involved in additional metabolic reactions other than phytohormone synthesis. Detoxification reactions and pathogen response may be good candidates for these additional functions.

Xanthine dehydrogenase

In plants, XDH (EC 1.1.1.204), but not the oxidase form, was identified in a variety of organisms and tissues (as reviewed by Nguyen, 1986). The enzyme was purified from nodules of bean (Boland, 1981) as well as from the green alga *Chlamydomonas reinhardtii* (Perez-Vicente *et al.*, 1992) and, recently, from wheat leaves (Montalbini, 1998). Plant XDH shows highest affinities for xanthine and hypoxanthine as substrate, but also accepts purines and pterins at much lower rates (Nguyen, 1986). Like the animal enzyme, plant XDH is homodimeric with a molecular mass of around 300 kDa (Montalbini, 1998). The gene for *Arabidopsis* XDH has been annotated, and from mammals, *Drosophila* and filamentous fungi a wealth of sequence information is available confirming the known biochemical relationship between XO and AO at the molecular level.

XDH is involved in ureide synthesis and purine catabolism. Legumes fall into two classes depending on the transport form of symbiotically fixed nitrogen, the amide-type synthesizes glutamine and asparagine, and the ureide-producers (e.g. soybean) form allantoin and allantoic acid via the oxidative breakdown of purines (Schubert and Boland, 1990) which requires less energy than in the former class (Triplett *et al.*, 1982). Purines are catabolized via hypoxanthine→xanthine→uric acid→allantoin→allantoic acid where XDH catalyses the first two steps. The

highest XDH activities found in higher plants were detected in nodules with levels 500–1000-fold higher than those encountered in leaves of legumes and non-legumes (Nguyen, 1986).

Plant XDH is very likely to produce superoxide radicals (Montalbini, 1992a), and the enzyme was shown to be involved in host-pathogen relationships between phytopathogenic fungi like *Uromyces* (Montalbini, 1992a) or *Puccinia* (Montalbini, 1992b) with legumes and cereals, respectively. XDH is also likely to be involved in senescence. Oxidative processes during senescence involve an increase in enzyme activities generating oxygen radicals and superoxide ions. In pea leaves, XDH activity was sharply increased in parallel with superoxide dismutase and other oxygen-related enzymes (Pastori and Rio, 1997). The question of what role XDH plays in activated oxygen metabolism during senescence, remains to be answered. The subcellular location of XDH is also not clear; it was suggested (Nguyen, 1986) that XDH might be associated with microbodies and later it was reported that pea leaf peroxisomes contain XDH activity which catabolizes xanthine to uric acid inside the organelles (Corpas *et al.*, 1997). On the other hand, immunocytochemistry of XDH in cowpea nodules demonstrated a cytoplasmic location (Datta *et al.*, 1991), and no targeting signal was found in the XDH sequence of *Arabidopsis* (R Mendel, unpublished data).

Sulphite oxidase

In plants, the existence of SO was a matter of controversy for a long time. During primary sulphate assimilation in chloroplasts, sulphate is reduced via sulphite to the organic sulphide which is used for cysteine biosynthesis (Leustek and Saito, 1999). However, it has been also reported that sulphite can be oxidized back to sulphate, for example, when plants were subjected to SO₂ gas (as reviewed in Heber and Hüve, 1998) or when isolated chloroplasts were fed with radioactively labelled sulphite (Dittrich *et al.*, 1992). Sulphite oxidizing activity was detected in the light and dark (Jolivet *et al.*, 1995). Thus SO would counteract sulphate assimilation, provided that it would be localized in the chloroplast. Very recently, work from this laboratory has identified SO (EC 1.8.3.1) as the fourth member of Mo-enzymes in plants (Eilers *et al.*, 2001). It is localized in peroxisomes and has a molecular mass of 90 kDa for the dimer.

Among eukaryotes, plant SO is the smallest Mo-enzyme known to date and the only one lacking redox-active centres other than Moco. The alignment of Moco domains of SOs from different sources with *Arabidopsis* SO and NR (Eilers *et al.*, 2001) demonstrates considerable overall homology, identifying these enzymes as members of a common family. Plant SO is conserved among higher plants as evidenced by the fact that antibodies raised against *Arabidopsis* SO detected a dominantly cross-

reacting protein band of about 45 kDa in a wide range of species belonging to a variety of both herbaceous and woody plants.

In mammals, SO has not only a Moco-domain but an additional haem domain at the N-terminus. Animal SO is localized in the intermembrane space of mitochondria (Cohen *et al.*, 1972) where electrons derived from sulphite are passed via the haem domain on to cytochrome *c*, the physiological electron acceptor. For plant SO it is very likely that it needs an electron acceptor protein of a redox potential similar to a haem. Taking into account that it is localized in the peroxisomes, the *b*-type cytochromes, recently described for plant peroxisomes (Lopez-Huertas *et al.*, 1999), are a likely candidate to fulfil the role of a physiological electron acceptor for plant SO. Since plant SO is not found in the chloroplasts one can assume that the function of SO is not related to the chloroplast-based sulphur assimilation pathway. Rather, it might be that plant SO has a sulphite-detoxifying function. For example, it has been shown, that peroxisomal catalase is inhibited already by low concentrations of sulphite (Veljovic-Jovanovic *et al.*, 1998).

Mo uptake into plants

How do Mo-enzymes acquire their catalytically important metal? Mo is a very rare element (Fortescue, 1992). Its oxidation state in soils varies from II to VI, but only the soluble Mo(VI) forms are available for plants. With decreasing pH, Mo can be adsorbed by soil colloids. Therefore more Mo is available for plants at higher soil pH values. The plants' requirement for Mo is very low (Gupta, 1997), but, nevertheless, Mo deficiency has been reported for many plant species including herbs, crops and trees (Gupta, 1997) and is mainly caused by the lack of NR catalysing the initial step in nitrate assimilation (Hewitt, 1983). Visible symptoms of Mo deficiency vary according to plant species and most often result in chlorosis or a yellowing of the leaves.

Until recently, a Mo transporter was not known in plants, so it was thought that molybdate was transported unspecifically by another anion transporter. This statement still holds true for the low affinity molybdate uptake because, in tomato plants, it was observed that phosphorus deficiency enhances uptake of radiolabelled molybdate up to five times, so that uptake of molybdate could occur via the phosphate uptake system (Heuwinkel *et al.*, 1992). Sulphate transporters are also likely candidates for low affinity Mo uptake into the cell because molybdate-uptake could be inhibited by large amounts of sulphate (Marschner, 1995). The co-transport of molybdate by a sulphate transport system was also observed in filamentous fungi (Tweedie and Segel, 1970). In *E. coli*, where a high affinity transport system of the ABC-type is known (summarized by Pau and Lawson, 2002), the sulphate

transporter was also shown to serve as a low-affinity molybdate transporter (Rosentel *et al.*, 1995; Grunden and Shanmugam, 1997). Very recently, mutant analysis in the green alga *Chlamydomonas reinhardtii* revealed for the first time a more detailed picture of molybdate uptake in eukaryotes (Llamas *et al.*, 2000). This algae has two molybdate uptake systems: one system is a high-affinity, low-capacity transporter that is insensitive to tungstate, but can be inhibited by 0.3 mM sulphate. The other system is a bulk transporter (low-affinity, high-capacity) that can be inhibited by tungstate, but not by sulphate. After its uptake into the plant, the soluble molybdate anion, which is the predominant aqueous species at pH values above pH 4.0, is found in phloem as well as in xylem and is assumed to be the major transport form in these two long-distance transport systems (Marschner, 1995).

Mo-cofactor

Early work with NR-deficient mutants of the filamentous fungi *Aspergillus nidulans* (Pateman *et al.*, 1964) and *Neurospora crassa* (Nason *et al.*, 1970) and of the higher plant *Nicotiana tabacum* (Mendel and Müller, 1976) revealed a novel mutant phenotype, namely the simultaneous loss of the two Mo-enzymes NR and XDH. Since Mo was the only common link between these two, otherwise very different, enzymes, it was suggested that both enzymes should share a common Mo-related cofactor, named molybdenum cofactor (Moco). Later Johnson *et al.* (1980) demonstrated that the organic component of Moco from different Mo-proteins is a unique pterin, which they called molybdopterin (MPT; cf. Fig. 1A). Obviously Mo itself seems to be biologically inactive; rather it has to be complexed with MPT in order to form the Moco and thereby become biologically active. The task of the cofactor is to position the catalytic metal Mo correctly within the active centre, to control its redox behaviour and to participate with its pterin ring system in the electron transfer to or from the Mo atom. The pterin, with its several possible reduction states as well as different structural conformations, could also be important for channelling electrons to other prosthetic groups (Kisker *et al.*, 1997b). X-ray crystallographic analyses of Mo-enzymes revealed that the cofactor is not located on the surface of the protein, but it is buried deeply within the interior of the enzyme and a tunnel-like structure makes it accessible to the appropriate substrates (Kisker *et al.*, 1998). Once Moco is liberated from the holoenzyme, it loses Mo and undergoes rapid and irreversible loss of function due to oxidation (Rajagopalan, 1996). The demolybdo-forms of Mo-enzymes are catalytically inactive (Kisker *et al.*, 1997b). Elevated amounts of the Mo-antagonist, tungsten, were shown to inhibit the activity of Mo-enzymes by replacing Mo as a ligand of MPT (Wray and Filner, 1970; Kisker *et al.*, 1997b), whereas in hyperthermophilic archaeobacteria, tungsten

seems to be the physiological ligand for MPT (Kletzin and Adams, 1996).

Moco mutants

For higher plants, a shortage of Mo in the soil or a mutational block of the cellular ability to use Mo, i.e. to synthesize MPT, to take Mo up into the cell or to bind it to MPT, lead to the loss of essential metabolic functions and cause, in most cases, the death of the plant. Moco-deficient plant mutants show a pleiotropic loss of all four Mo-enzyme activities NR, XDH, AO, and SO. Moco-mutants have been described in numerous higher plants, for example, in tobacco (Mendel and Müller, 1976, 1978), *Nicotiana plumbaginifolia* (reviewed by Müller and Mendel, 1989) and barley (reviewed by Kleinhofs *et al.*, 1989), and they were also found in green algae such as *Chlamydomonas reinhardtii* (reviewed by Fernandez and Cardenas, 1989). In the true diploid tobacco species *N. plumbaginifolia*, mutants in six Moco-specific genetic loci (*cnxA–cnxF*) were described, showing a similar morphology strongly deviating from that of the wild type: stunted growth, chlorosis of leaves, as well as small, narrow and crinkled leaves (Gabard *et al.*, 1988). For *Arabidopsis* five Moco loci (designated *chl*) were characterized (Crawford, 1992) which, however, turned out to be leaky with background activities in the range of 10% which hampered their further analysis. The multitude of Moco-specific loci led to the conclusion that Moco biosynthesis is a complex and probably ancient pathway with several gene products involved (Müller and Mendel, 1989; Mendel, 1992, 1997; Rajagopalan and Johnson, 1992). In pre-genomic times, the detailed mutant characterization contributed substantially to an understanding of the genetics and biochemistry of Moco in bacteria, plants, fungi, insects, and mammals. These analyses allowed a more precise description of the impairment caused by a given mutation in the biosynthetic pathway of Moco. By measuring intracellular amounts of Mo it could be ruled out that plant Moco mutants are defective in Mo uptake (Mendel *et al.*, 1984). Among the Moco mutants, one mutant type was always partially repairable by growth on high-molybdate medium so that it was assumed that the gene product of the molybdate-repairable locus should be involved in transferring or inserting Mo into the cofactor. Molecular analyses proved this assumption to be correct (see below). Mutants in the loci non-repairable by molybdate were interpreted to be defective in the biosynthesis of MPT itself.

Moco biosynthesis

The MPT-structure of Moco is conserved in all organisms, hence it was tempting to conclude that perhaps also (part of) the biosynthetic pathway for Moco could be similar in all organisms (Mendel,

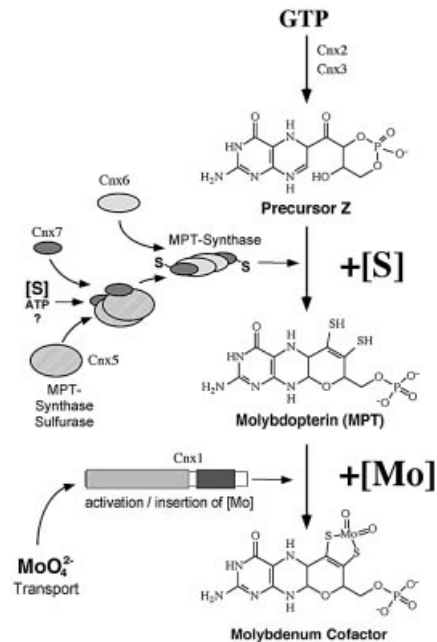


Fig. 2. Model for the biosynthetic pathway of Moco in *Arabidopsis thaliana*. Moco biosynthesis proceeds in three steps: (1) conversion of GTP to precursor Z, (2) insertion of sulphur and formation of molybdopterin, (3) insertion of molybdenum and formation of Moco. The proteins involved are named according to the Cnx nomenclature (Caboche *et al.*, 1994). The role of Cnx4 is not as yet clear, therefore it is not included in this model.

1992). Several approaches have been successfully used to clone eukaryotic genes involved in Moco biosynthesis: functional complementation of *E. coli* Moco mutants, generation of Moco-defective mutants by tagging, cloning via 'expressed sequence tags' by searching for homologies on a protein level, exploiting protein-protein interactions by using the yeast two-hybrid system. On the amino acid level significant homologies (30–40% identity) do emerge between bacterial and eukaryotic Moco biosynthesis proteins. It turned out that nearly all *E. coli* Moco proteins have counterparts in plants. Six genes were cloned in *Arabidopsis thaliana* and, according to international rules, the Moco genes are designated *cnx* (Caboche *et al.*, 1994) which is a mnemonic for cofactor for nitrate reductase and xanthine dehydrogenase (Pateman *et al.*, 1964; Mendel and Müller, 1978). Researchers from this laboratory recombinantly expressed the proteins encoded by the six *Arabidopsis cnx* genes, purified them and started with their enzymological characterization. Comprehensive analyses of these proteins, their genes and plant mutants involving molecular, genetic, biochemical, and structural studies and a comparison of these data with the model for Moco biosynthesis in *E. coli* (Rajagopalan, 1996), led to a picture of Moco biosynthesis consisting of three stages in plants.

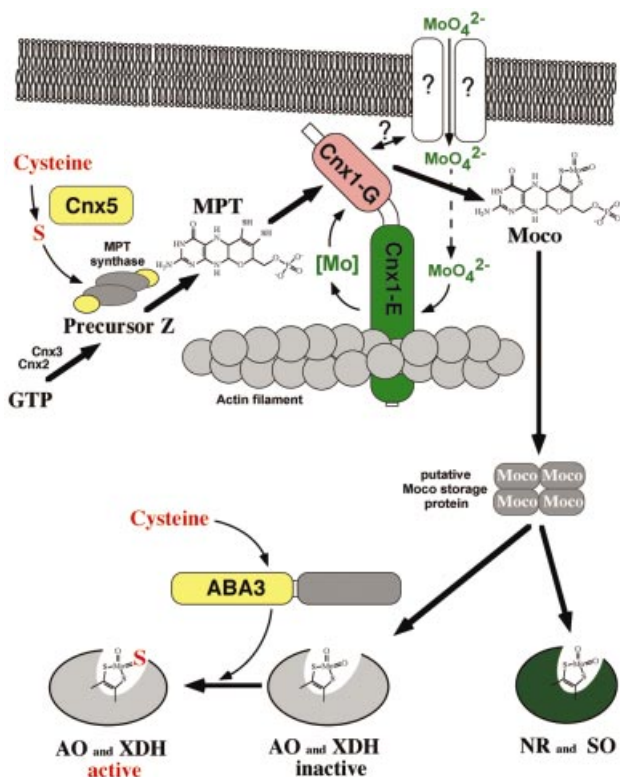


Fig. 3. Model for Moco biosynthesis in plant cells according to Schwarz *et al.* (2000) and Bittner *et al.* (2001). Cnx2 and Cnx3 converts GTP to precursor Z. MPT synthase consisting of its subunits Cnx6 and Cnx7, inserts sulphur into precursor Z and converts the precursor into MPT. MPT synthase is sulphurated by Cnx5. Subsequently, MPT is bound to Cnx1 being located under the plasmalemma and bound to an actin filament. The interaction of Cnx1 with an integral membrane protein is based on the function described for the Cnx1-homologous animal protein gephyrin in neuroreceptor anchoring (Ramming *et al.*, 2000) and needs to be shown. A putative molybdate-anion channel is proposed that interacts with Cnx1 to facilitate molybdate channelling to the E domain of Cnx1. This domain generates an activated form of Mo that is incorporated by the C-terminal domain into the bound MPT. MPT is highly sensitive to oxidation, therefore rapid conversion of precursor Z via MPT to Moco in a multienzyme complex anchored by Cnx1 on the cytoskeleton is suggested. Finally, Moco is bound by a putative Moco storage protein that supplies the cofactor to the Mo-enzymes. NR and SO need no further modification of Moco, while AO and XDH need a final step of maturation: the sulphurase ABA3 replaces an oxygen atom by a sulphur thereby activating the enzymes.

Stage 1: During the first step, a guanosine-X-phosphate derivative (probably GTP) is transformed into a sulphur-free pterin compound, the precursor Z, already possessing the Moco-typical four carbon side chain (Fig. 2). This step is catalysed by the proteins Cnx2 and Cnx3. Their genes have been cloned by functional complementation of the corresponding *E. coli* Moco mutants (Hoff *et al.*, 1995), which indicates that the function of this pair of proteins has been strongly conserved during evolution, but nothing is known about the mechanism of their functioning.

Stage 2: In the second step of Moco biosynthesis, two sulphur atoms have to be incorporated into precursor Z.

This reaction is catalysed by the enzyme MPT synthase, a heterotetrameric complex of two small (Cnx7) and two large (Cnx6) subunits, that stoichiometrically converts precursor Z into MPT. The sulphur is bound to the C-terminus of Cnx7 as thiocarboxylate. After MPT synthase has transferred the two sulphurs to precursor Z, it has to be resulphurated by the sulphurase Cnx5 in order to reactivate the enzyme for the next reaction cycle of precursor Z conversion. The identity of the donor for the reactive mobile sulphur is as yet unknown in plants, but there are indications that make cysteine a likely candidate.

Stage 3: After synthesis of the MPT moiety, the chemical backbone has been built for binding and co-ordination of the Mo atom. In the last step, therefore, Mo has to be transferred to MPT in order to form Moco. Mutants defective in this step produce MPT and can be partially repaired by growing them on high-molybdate medium. These mutants are defective in the gene *cnx1*. It was isolated from *Arabidopsis* by functional complementation of the corresponding *E. coli* mutant (Stallmeyer *et al.*, 1995) and was mapped (Stallmeyer *et al.*, 1995) to the molybdate-repairable *chl-6* locus (Braaksma and Feenstra, 1982). Cnx1 is a two-domain protein where the N-terminal domain is essential for generating an activated form of Mo that is incorporated by the C-terminal domain into the bound MPT (Schwarz *et al.*, 1997, 2000). Finally, Cnx1 is essential for stabilizing the newly formed Moco (Kuper *et al.*, 2000). Recently, it was demonstrated that Cnx1 is able to bind to actin filaments via its N-terminal domain (Schwarz *et al.*, 2000). What could be the functional significance of cytoskeleton binding of Cnx1 in terms of Moco biosynthesis? It was assumed that, during evolution, it became important to facilitate substrate-product flow which could result in microcompartmentalization of a hypothetical Moco-biosynthetic multienzyme complex ensuring the fast and protected transfer of the labile intermediates within the reaction sequence from GTP to Moco (Fig. 3, upper part). Therefore anchoring to submembranous cellular structures like the cytoskeleton might help organizing and stabilizing such a biosynthetic machinery and would bring it close to the putative molybdate-anion channel providing the metal for Moco synthesis. For details see the recent review of Mendel and Schwarz (Mendel and Schwarz, 2002).

Storage and insertion of molybdenum cofactor into Mo-enzymes

The availability of sufficient amounts of Moco is essential for the cell to meet its changing demand for synthesizing Mo-enzymes. In particular, the diurnal variation in the amount of NR protein requires a flexible regulation of Moco synthesis. Here, the existence of Moco-storage proteins would be a good way to buffer supply and demand

of Moco. In fact, Moco-binding proteins were described for seeds from *Vicia faba* (Kalakoutskii and Fernandez, 1996), wheat and barley (Alikulov and Schiemann, 1985; Vunkova-Radeva *et al.*, 1988) and for *Chlamydomonas reinhardtii* (Witte *et al.*, 1998). Their detailed function and reaction within the cell, however, is still unknown.

Insertion of Moco into Mo-enzymes is not understood. Using a defined *in vitro*-system it was shown that human apoSO can directly incorporate Moco (Leimkühler and Rajagopalan, 2001). However, for inserting of Moco into the target apo-enzymes as it occurs in the living cell either (still unknown) chaperones would be needed or the Moco storage/binding proteins could become involved at this stage. For some bacterial Mo-enzymes, system-specific chaperones are required for Moco insertion and protein folding, e.g. NarJ for *E. coli* NR (Blasco *et al.*, 1998) and XDHC for *Rhodobacter capsulatus* XDH (Leimkühler and Klipp, 1999).

Terminal activation of Mo-enzymes by sulphuration

Figure 3 depicts the single steps of Mo-enzyme formation in plants. Moco is the endproduct of the Moco biosynthetic pathway. Subsequently, a putative Moco-binding/storage protein could distribute Moco to the apoproteins of Mo-enzymes thus taking over the task to buffer supply and demand of Moco. Finally, Moco is inserted into the apoproteins thereby converting them to holo-enzymes. The four Mo-enzymes in plants can be subdivided into two groups: NR and SO possess a dioxo-Mo centre and are activated by insertion of Moco. XDH and AO, however, require a separate final step of maturation after insertion of Moco. XDH and AO have a mono-oxo Mo-centre and need the addition of a terminal inorganic sulphur to the Mo-centre in order to gain enzymatic activity. This sulphur ligand does not originate from the apoprotein nor does it come from the Moco moiety. For rat and fly (Wahl *et al.*, 1982; Wahl and Rajagopalan, 1982) it was demonstrated that, *in vitro*, this sulphur can be spontaneously lost or can be removed from AO/XDH by cyanide treatment generating an inactive enzyme. The reaction, however, is reversible and the enzyme can be reactivated by sulphide-treatment under reducing conditions. *In vivo*, this terminal sulphur has to be added by a separate enzymatic reaction. Bittner *et al.* (2001) identified the protein ABA3 as Moco-sulphurase catalysing the activation of enzymes with a mono-oxo Mo centre by insertion of the cyanolysable sulphur into the active centre (Fig. 3, lower part). ABA3 performed this S-transfer with assembled AO (Bittner *et al.*, 2001), therefore at least under *in vitro* conditions ABA3 is able to attach sulphur to Moco already bound to the enzyme. The N-terminus of ABA3 shares significant homologies to the bacterial sulphurase NifS. It is proposed that in a pyridoxal phosphate-dependent

mechanism of (*trans*)sulphuration an ABA3-bound per-sulphide, resulting from the desulphuration of free L-cysteine to L-alanine, is likely to be transferred to the Mo-centre. The C-terminal domain is probably responsible for mediating the contact between XDH/AO and the *trans*-sulphurase-domain of ABA3 (Bittner *et al.*, 2001).

Mutants defective in the terminal Moco-sulphurase were described for fly (Finnerty *et al.*, 1979; Wahl *et al.*, 1982), *A. nidulans* (Scazzocchio, 1973) and several plants (Leon-Kloosterziel *et al.*, 1996; Leydecker *et al.*, 1995; Marin and Marion-Poll, 1997). They were lacking both XDH and AO activities but retained their activities of enzymes with a dioxo Mo centre. Due to the lack of AO the plant mutants had only residual levels of ABA and this was exactly the phenotype that led to the discovery of these mutants and to their designation. The terminal sulphuration step seems to be an interesting regulatory switch-point because the activity of ABA3 could control the amount of functional AO/XDH molecules in the cell that, in turn, would control cellular levels of, for example, ABA. In fact, transcription of the *aba3* gene was found to be inducible by drought-stress and by cold-stress in *Arabidopsis* (Xiong *et al.*, 2001; Bittner *et al.*, 2001).

Concluding remarks

The understanding of the role and the function of molybdenum in plants is progressing rapidly. Now that all relevant genes are cloned and the basic function of the proteins encoded are known, research concentrates both on the detailed enzymology of Moco biosynthesis and on studying regulation and structure–function relationships of Mo-enzymes. The crystallization of plant Mo-enzymes, as well as Moco biosynthesis proteins, is underway. There is a wealth of physiological data for NR, but not for SO, XDH and AO and there are, as yet, a number of open questions. What additional metabolic reactions are catalysed by the members of the AO family? What is the exact role of SO and why is it localized in the peroxisomes? What is the role of XDH in reactive oxygen metabolism and in plant defence? How is the molybdate transporter organized and what genes code for it? How is the multienzyme complex for Moco biosynthesis organized? How is Moco biosynthesis regulated to meet the changing demands of the cell for Moco? The coming years will bring insight into the integration and (perhaps unexpected) regulatory connections of Moco-biosynthesis and Mo-enzymes within the metabolic and physiological network of the plant cell.

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