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Review

Molybdenum cofactor biosynthesis in plants and humans

Ralf R. Mendel[a,*], Günter Schwarz[b]

[a] Institute of Plant Biology, Braunschweig University of Technology, 1 Humboldt Street, 38106 Braunschweig, Germany
[b] Institute of Biochemistry, University of Cologne, 47 Zuelpicher Street, 50674 Cologne, Germany

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ABSTRACT

The transition element molybdenum (Mo) needs to be complexed by a special cofactor in order to gain catalytic activity. With the exception of bacterial Mo-nitrogenase, where Mo is a constituent of the FeMo-cofactor, Mo is bound to a pterin, thus forming the molybdenum cofactor Moco, which in different variants is the active compound at the catalytic site of all other Mo-containing enzymes. The biosynthesis of Moco involves the complex interaction of six proteins and is a process of four steps, which also requires reducing equivalents, iron, ATP and probably copper. After its synthesis, Moco is distributed to the apoproteins of Mo-enzymes by Moco–carrier/binding proteins that also participate in Moco-insertion into the cognate apoproteins. A deficiency in the biosynthesis of Moco has lethal consequences for the respective organisms. In humans, Moco deficiency is a severe inherited inborn error in metabolism resulting in severe neurodegeneration in newborns and causing early childhood death. Due to our better understanding of the chemistry of Moco synthesis, a first therapy has been brought to the clinic.

1. Introduction

It has been long known that the rare transition element molybdenum (Mo) is an essential micronutrient for plants, animals and microorganisms. Surprisingly, Mo itself is catalytically inactive in biological systems until it is complexed by special cofactors. One type of cofactor is the iron–sulfur cluster-based iron–Mo-cofactor that is unique to the Mo nitrogenase (which will be reviewed in detail by Ribbe [1]). The other type of cofactor is the pterin-based Mo-cofactor (Moco) that in different variants forms part of the active centers of all Mo-enyzmes in living organism. In this review we will focus on higher organisms (eukaryotes) and will follow the way that Mo takes from uptake into a cell, via formation of the Moco and its storage, to its insertion into mature apo-metalloenzymes which also requires final modification. Among higher organisms,

Abbreviations: cPMP, cyclic pyranopterin monophosphate; Mo, molybdenum; Moco, molybdenum cofactor; MPT, molybdopterin; SAM, S-adenosyl methionine.

* Corresponding author. Tel.: +49 531 391 5870; fax: +49 531 391 8128.
E-mail addresses: r.mendel@tu-bs.de (R.R. Mendel), gschwarz@uni-koeln.de (G. Schwarz).

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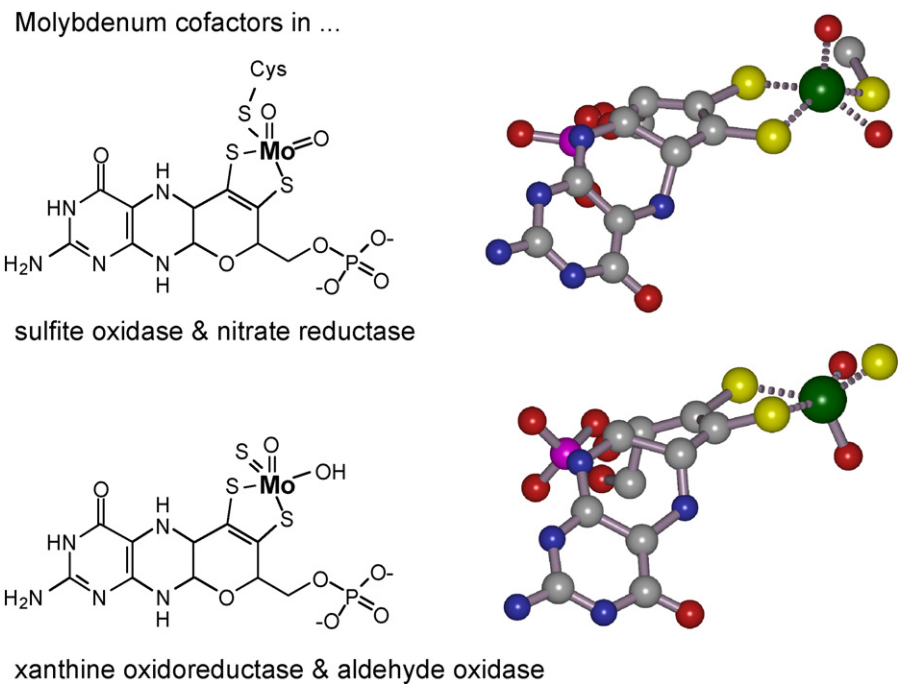


Fig. 1. Molybdenum cofactors in enzymes of the sulfite oxidase and xanthine oxidase family. Chemical structure and three-dimensional projection of Moco (ball-and-stick mode) as seen in the crystal structure of *Pichia angusta* nitrate reductase Mo domain [14] and bovine xanthine oxidase [89]. Both types of cofactors show a pyramidal geometry of the Mo center with one pointing up-wards and one pointing down-wards with respect to the apical oxygen.

most of this knowledge derives from studies in plants and humans which may be surprising – but the yeast *Saccharomyces cereviseae* as model organism plays no role in Mo research as it belongs to those organisms that do not contain Mo-enzymes while many other yeasts such as *Pichia pastoris* do need Moco. Mo-enyzmes and Moco are found in all kingdoms of life, and amongst all organisms genetically investigated approximately two thirds require Mo for life (Gladyshev [2]). For higher organisms, Hille [3] will discuss the most prominent Mo-enzymes in detail. In brief, these enzymes are: (1) sulfite oxidase, which catalyzes the final step in the degradation of sulfur-containing amino acids and is involved in detoxifying excess sulfite; (2) xanthine dehydrogenase, which is involved in purine catabolism and reactive oxygen production; (3) aldehyde oxidase, which oxidizes a variety of aldehydes and is essential for the biosynthesis of the stress hormone abscisic acid in plants; (4) nitrate reductase in autotrophic organisms, which catalyzes the key step in inorganic nitrogen assimilation; (5) mitochondrial amidoxime reductase which has a detoxifying function. Structure and function of all five enzymes are reviewed by Hille [3].

2. Molybdenum uptake

Organisms take up Mo in the form of its oxyanion molybdate. It requires specific uptake systems to scavenge molybdate in the presence of competing anions. These Mo uptake systems were studied in detail in bacteria while in higher organisms only recently first molybdate-transporting proteins have been identified in algae and plants (see review by Hagen [4]). Two proteins (MOT1 and MOT2) belonging to the large sulfate carrier superfamily were shown to transport molybdate with high affinity across cellular membranes [5–7]. Unexpectedly, none of them was found to reside in the plasmamembrane surrounding the cell. Contradictory reports localized them to the endo-membrane system [5] or to the mitochondrial envelope [7] which requires further studies, as at least for the mitochondria molybdate uptake should not be required. It is likely that additional transporters, not only in plants but also in animals, will be discovered soon.

3. The molybdenum cofactor

Once having entered the cell, Mo has to be attached to its cofactor scaffold thereby converting to Moco and gaining biological activity. Nason et al. [8] provided first biochemical evidence for a cofactor common to all Mo-enzymes that could be removed as low molecular weight fraction from denatured Mo-enzymes of mammalian, plant and bacterial origin and subsequently incorporated into a cofactor-free apo-nitrate reductase thus activating the enzyme. Later, the chemical nature of Moco was elucidated by the work of Rajagopalan and Johnson [9] (Fig. 1). As Moco turned out to be very labile and sensitive to air-oxidation, its degradation and oxidation products were used to uncover its nature. By analysis of two oxidation products the pterin nature of Moco and its C6 substitution with a unique four-carbon side chain were identified [10]. Due to the sensitivity of Moco to sulfhydryl reagents and its increased stability in the presence of reducing reagents, the presence of sulfur atoms in the cofactor was proposed [11]. Later, the coordination of Mo via an ene-dithiolate group located within the four-carbon side chain of the cofactor was demonstrated by carbamidomethylation of Moco [12]. Finally crystal structures of Mo-enzymes confirmed this core structure and showed the existence of a third pyrano ring between the OH-group at C3′ of the side chain and the pterin C7 atom. Once the pyrano ring is closed, a fully reduced hydrogenated pterin is formed. Because of the unique nature of the pterin in Moco, the metal-free form of the cofactor is called *m*olybdo*pt*erin or *m*etal-binding *pt*erin (MPT), the latter reflects the fact that not only Mo but also tungsten can be coordinated by this pterin scaffold.

What is the task of the pterin moiety of Moco? Obviously the pterin functions in binding and coordinating the catalytic metal correctly within the active center of a given Mo-enzyme and depending on the class of Mo-enzyme, Mo can have different ligands (cf. review by Hille [3]). Another possible role of the pterin moiety could be control of the redox behavior of the Mo atom. In addition, the pterin might also participate in the electron transfer to or from Mo via the delocalized electrons within the pterin.

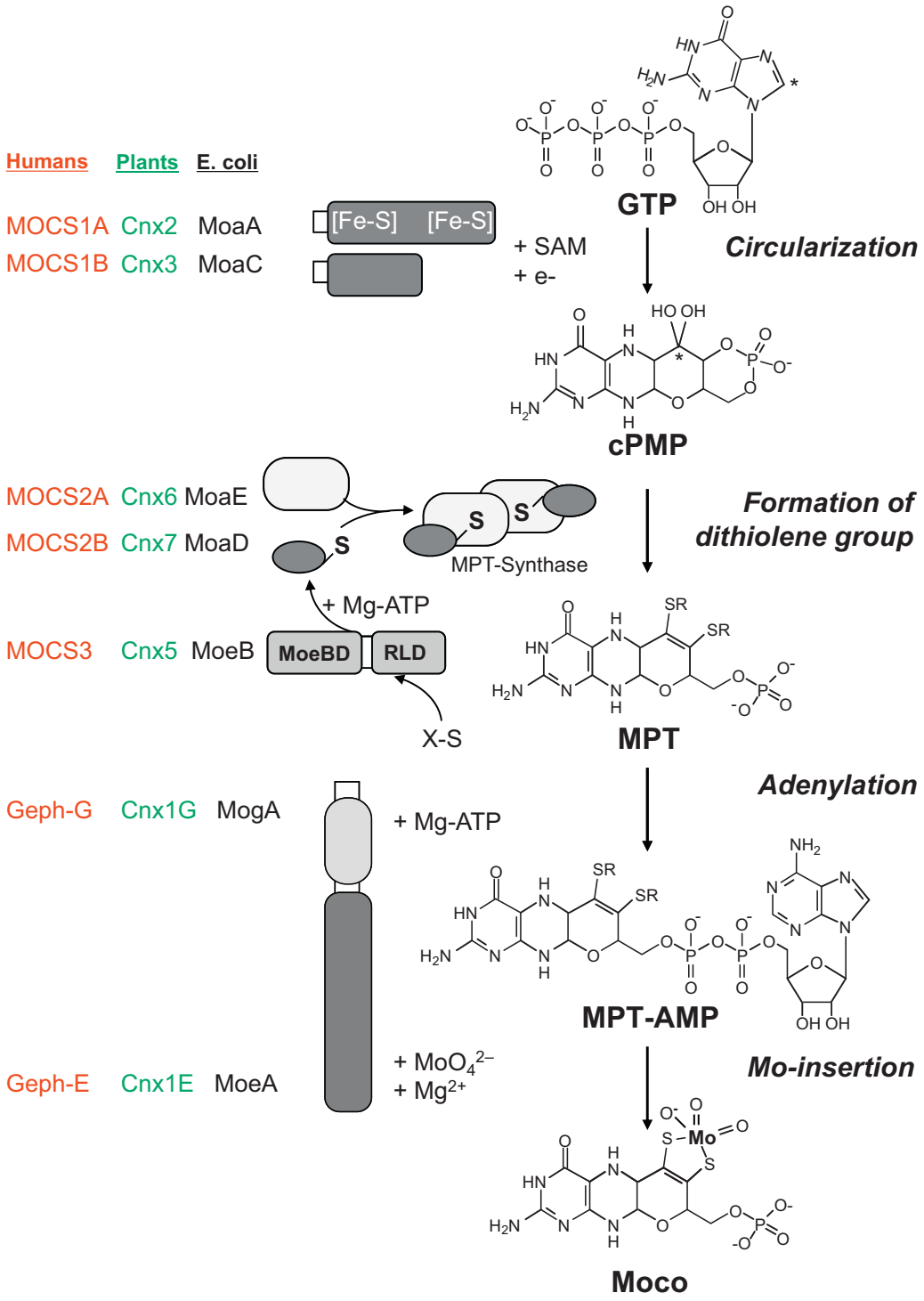


Fig. 2. Biosynthesis of molybdenum cofactor. The names for the proteins from plants (green), humans (red) and *E. coli* (black) catalyzing the respective steps are given. [Fe–S], Fe–S clusters; SAM, S-adenosyl methionine, MoeBD, MoeB-like domain; RLD, rhodanese-like domain. In GTP, the C8 atom of the purine is labeled with a star. This carbon is inserted between the 2′ and 3′ ribose carbon atoms, thus forming the new C1′ position in the four-carbon side chain of the pterin (labeled with a star in cPMP). The in vivo source of sulfur (X-S) for Cnx5 and MOCS3 is not known yet. Steps three and four in plants and humans are catalyzed by the individual domains of Cnx1 (G and E) or Gephyrin (G and E). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Moco is not located on the surface of the protein, rather X-ray crystallographic analyses of Mo-enzymes revealed that the cofactor is buried deeply within the interior of the enzyme and a tunnel-like structure makes it accessible to the appropriate substrates ([13,14] and review by Dobbek [15]). During its life time, the Mo-enzyme does not liberate Moco. In vitro, however, one can remove Moco from the holoenzyme whereafter Moco loses Mo and undergoes rapid and irreversible loss of function due to oxidation. To this end there are no indications for a Moco recycling mechanism in the cell.

In bacteria, the MPT-based core moiety of Moco can be further modified to form bis-MPT equivalents or dinucleotide variants of Moco (cf. reviews by Leimühler [16] and by Weiner [17]).

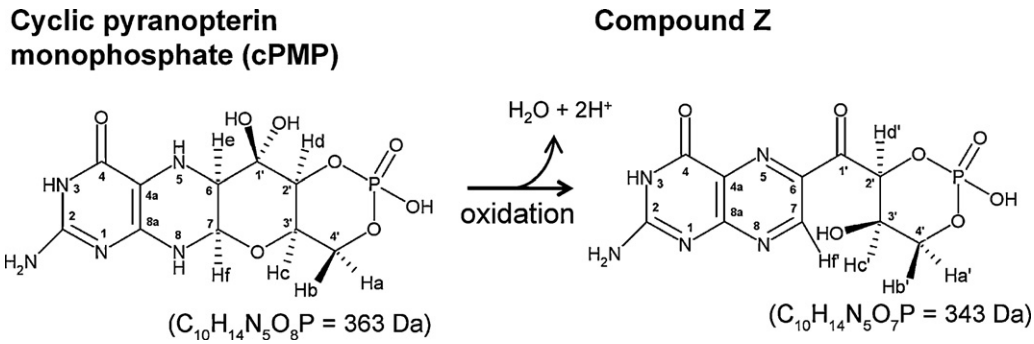


Fig. 3. Oxidation of cPMP to compound Z. Note, that a two-electron oxidation of the pterin results in a fully oxidized pterin coupled to the opening of the pyranoring and water release thus forming a keto function in the side chain [33].

4. Moco biosynthesis

The genetic analysis of Moco-deficient mutants in a given organism ranging from bacteria to humans resulted in the identification of several gene loci being involved in Moco biosynthesis. This fact together with the conserved structure of Moco provided the basis to propose an evolutionarily conserved multi-step biosynthetic pathway for Moco [18]. The first model for Moco biosynthesis was presented by Rajagopalan and Johnson [9] for the bacterium *Escherichia coli*. Later studies of Moco biosynthesis uncovered a more complex picture of this pathway in higher organisms [19,20] where molecular, biochemical and genetic analyses of Moco mutants were most advanced in plants. These results formed the basis to decipher Moco biosynthesis also in humans (summarized in [21]) and it turned out that the pathways of Moco biosynthesis showed many similarities in both organisms and therefore we will compare them whenever appropriate.

In all higher organisms studied so far, Moco is synthesized by a conserved biosynthetic pathway that can be divided into four steps, according to the biosynthetic intermediates cyclic pyranopterin monophosphate (cPMP), MPT, adenylated MPT (MPT–AMP), and Moco (Fig. 2). Always six gene products (=proteins) catalyzing Moco biosynthesis have been identified in plants [19], fungi [22] and humans [23–25]. These genes are homologous to their counterparts in bacteria. Genes and the encoded proteins were named in plants according to the *cnx* nomenclature (*c*ofactor for *n*itrate reductase and *x*anthine dehydrogenase). For human Moco synthetic genes, a different MOCS (*mo*lybdenum *co*factor *s*ynthesis) nomenclature was introduced, and the names for both the plant and human proteins are given in Fig. 2. A third nomenclature is used among bacteria. For comparison, Fig. 2 also shows the names of the corresponding bacterial proteins so that the reader has a cross-reference to the review by Leimkühler [16]. Now, we will discuss the individual steps of Moco biosynthesis.

4.1. Chemical properties and structure of cPMP

The first relatively stable intermediate in Moco biosynthesis is cyclic pyranopterin monophosphate (cPMP; Fig. 2) which was first isolated and preliminarily characterized more than 17 years ago and initially named precursor Z [26]. In its first description, it was presented as quinoidal dihydropterin with a cyclic phosphate and found to be relatively stable against oxygen at low pH with an estimated half-life of several hours [26]. With the clarification of the chemical structure of Moco by crystal structures of Mo-enzymes from bacteria and higher organisms the question arose at which stage the formation of the pyrano ring occurs. For xanthine dehydrogenase a ring closure during Moco insertion has been proposed [27] and in the crystal structure of bacterial nitrate reductase A one of the two MPT equivalents has been observed in the ring-opened bicyclic form [28] indicating a possible role of the pterin's redox state in catalysis.

Like other pterins, cPMP oxidizes under aerobic condition into its stable oxidation product compound Z [26]. Based on a single-step two-electron oxidation of cPMP (Fig. 3) the dihydrostate of the pterin has been proposed [26] which formally would fit with a pyranopterin structure. In contrast to cPMP, dihydropterins are characterized by a high sensitivity to oxygen with half-lives of minutes [29] while tetrahydropterins such as biopterin are more stable at physiological pH ($t_{1/2}$ = 10–20 min) [30]. Studies with different synthetic pyranopterins demonstrated a remarkable increase in stability of pyranopterins in comparison to dihydro and tetrahydropterins [31,32], which fits much better with the observed stability of cPMP. The chemical structure of cPMP was clarified using high resolution mass spectrometry and ^{1}H NMR spectroscopy [33]. Key to these studies was the fermentation and purification of cPMP from an *E. coli* strain with a mutational block in the conversion of cPMP and simultaneous overproduction of the bacterial cPMP-synthesizing enzymes MoaA and MoaC. Consistent with the physico-chemical properties of cPMP, a pyranopterin structure with a geminal diol in the C1′ position and terminal cyclic phosphate was found (Fig. 3). Therefore the molecule (previously known as precursor Z) was named 1′,1′-dihydroxy 2′,4′ cyclic *p*yranopterin *m*onophos*p*hate (cPMP). The pyranopterin structure perfectly fits with the two-electron oxidation of cPMP to compound Z and demonstrates that Moco is synthesized as pyranopterin.

The geminal diol served as matter of discussion as earlier reports have not seen such a structure [34]. Also a recent crystal structure of MPT synthase (see below) in complex with cPMP could not clarify the chemistry of the side chain. Recently, we were able to purify cPMP quantities large enough to run ^{13}C NMR spectra, which confirmed previous structural assignments [33] (Santamaria-Araujo and Schwarz, unpublished results). Geminal diols are rare but found also in other pathways, such as the dihydroxy acetone phosphate and glyceraldehyde phosphate in glycolysis [35] and 2′-carboxy-3-keto-arabinitol-1,5-bisphosphate in carbon fixation cycle [36,37]. In both examples, involved molecules have a structure similar to cPMP. It is tempting to speculate that the electronic state of the pyrazine ring is the main contributor to form and stabilize the geminal diol in the absence of an enzyme. In summary, the geminal diol of cPMP might also have a protective function for cPMP and could direct the subsequent sulfur transfer reaction. As geminal diols are only observed in a strong electrophilic environment, opening of either the pyrano or cyclic phosphate should result in a loss of the hydration at C1′ (see below), which would explain, why the oxidation product, compound Z (Fig. 3), cannot be reduced back to cPMP [26].

4.2. Step 1: conversion of GTP into cPMP

Similar to the biosynthesis of other pteridines, Moco synthesis starts with guanosine 5′-triphosphate (5′-GTP) (Fig. 2), which is converted by a complex reaction sequence into cPMP [38]. Different from the other pteridine pathways (producing three-carbon side chains), MPT is unique in having a four-carbon side chain. By combining labeling studies with ^{1}H NMR analysis it has been confirmed that during the conversion of GTP into cPMP each carbon atom of the ribose and the ring atoms of the guanine are incorporated into cPMP [39,40]. The underlying mechanism, which still needs further investigation, involves a complex rearrangement reaction in which the C8 atom (labeled with a star in Fig. 2) of the purine is inserted between the 2′ and 3′ ribose carbon atoms, thus forming the new C1′ position in the four-carbon side chain of the pterin.

As first intermediate, cPMP is still sulfur-free but has already the tricyclic pyranopterin structure similar to the mature cofactor. In all organisms, the conversion of GTP to cPMP is catalyzed by two proteins, one of them (Cnx2 in plants, MOCS1A in humans) belongs to the superfamily of S-adenosyl methionine (SAM)-dependent radical enzymes [41]. Members of this family catalyze the formation of protein and/or substrate radicals by reductive cleavage of SAM by a [4Fe–4S] cluster [42]. MOCS1A is a protein containing two oxygen-sensitive Fe–S clusters each coordinated by only three cysteine residues [43]. The N-terminal [4Fe–4S] cluster, present in all radical SAM proteins, binds SAM and carries out the reductive cleavage of SAM to generate the 5′-deoxyadenosyl radical, which subsequently initiates the transformation of 5′-GTP bound through the C-terminal [4Fe–4S] cluster. The bacterial homolog (MoaA–protein) has been crystallized and its complex reaction mechanism [38,44,45] is described in [16]. The function of the second protein involved in catalyzing step 1 (i.e., Cnx3 in plants and MOCS1B in humans) is yet unknown but it is believed that it participates in pyrophosphate release upon the rearrangement reaction [38]. Since some of the SAM-dependent radical enzymes require another protein onto which the radical is transferred, it is tempting to speculate that Cnx3 and MOCS1B might have a similar function. Step 1 of Moco biosynthesis seems to be highly conserved between higher organisms and bacteria because the plant, human and fungal genes are able to replace the function of their bacterial homologs MoaA and MoaC in bacteria, respectively.

4.3. cPMP synthesis takes place in mitochondria

Both proteins catalyzing step 1 of Moco biosynthesis (Fig. 2) carry N-terminal extensions with putative targeting motifs for mitochondrial transport. Using antibodies directed against the two plant proteins Cnx2 and Cnx3 and organellar preparations of plant cells showed that both proteins were only detected in the mitochondrial fraction, but they could not be detected in either the cytosol or the chloroplasts [46]. Sub-fractionations of mitochondria showed that Cnx2 and Cnx3 were most abundant in the matrix fraction. In fact, the localization of Cnx2 and Cnx3 in this particular organelle appears reasonable from a physiological point of view as the mitochondrial matrix basically is equipped to provide (i) 5′-GTP as substrate for cPMP synthesis, (ii) Fe–S clusters as the essential prosthetic group for Cnx2 [47] and (iii) a reducing environment for stabilization of these oxygen-sensitive clusters bound to Cnx2.

As cPMP synthesis takes place in mitochondria while all subsequent steps of Moco biosynthesis have been demonstrated to be localized in the cytosol [48–50], export of cPMP from the mitochondria into the cytosol is required to allow further processing to Moco. For this task the transporter protein Atm3 [46], which is localized in the inner membrane of mitochondria and belongs to the family of ATP-binding cassette (ABC) transporters, is required. Surprisingly, Atm3 functions also in exporting another – however as yet unidentified – compound essential for Fe–S cluster synthesis in the cytosol [51]. However, when the Atm3 exporter is mutated, cPMP accumulates within mitochondria and the cytosol becomes short of cPMP with the consequence that Moco levels and Mo enzyme activities decrease in the cell. The precise role of Atm3 is still unknown. Also human cells possess this transporter protein, which is named ABCB7.

4.4. Step 2: synthesis of the MPT dithiolate

In the second stage of Moco biosynthesis sulfur is transferred to cPMP to form the MPT dithiolate (Fig. 2). This reaction is catalyzed by the enzyme MPT synthase, a heterotetrameric complex of two small (Cnx7 and MOCS2B, respectively) and two large (Cnx6 and MOCS2A, respectively) subunits that stoichiometrically converts cPMP into MPT. Each of the small subunits within the tetrameric enzyme complex carries a single sulfur atom as thiocarboxylate at the C-terminus. Among all small subunits so far analyzed from diverse species, the C-terminal region is highly conserved which includes a terminal double glycine. The functional importance of the thiocarboxylation has been shown for the bacterial [52] as well as human proteins [53].

The crystal structure of *E. coli* MPT synthase shows that the thiocarboxylated C-terminus is deeply inserted into the large subunit to from the active site of MPT synthase [54]. Bacterial MPT synthase was found to be an elongated protein complex with two clearly separated active sites [54] and two theories were postulated for the reaction mechanism of MPT synthase [34,52]. Both models have in common that the two sulfur atoms are not simultaneously transferred to cPMP, rather the sulfur atoms become sequentially inserted starting with C2′ of cPMP with the consequence that a monosulfurated reaction intermediate will occur [34]. A final clarification whether the intermediate will be transferred within the MPT synthase to the other active site [52] or whether the enzyme dissociates to host another sulfurated small subunit [34] remains to be given. Again, like in step 1, also the reaction mechanism of MPT synthase is conserved between bacteria and higher organisms as, at least for the large subunits, proteins can be exchanged between organisms. Furthermore, the use of an in vitro thiocarboxylation system [52] made it possible to study human MPT synthases carrying mutations that have been found in patients suffering from Moco deficiency [55] (see below).

4.5. Source of dithiolate sulfur atoms

After MPT synthase has transferred the two sulfur atoms to cPMP, it has to be re-sulfurated in order to regenerate the enzyme for the next reaction cycle of cPMP conversion. This separate resulfuration is catalyzed by the enzyme MPT-synthase sulfurase (Cnx5 and MOCS3, respectively), and at this stage the sulfur transfer reaction in higher organisms appears to involve different protein components as compared to most bacteria. In *E. coli*, the MoeB protein activates the small subunit of MPT-synthase by adenylation of the C-terminal glycine followed by the interaction with another enzyme (a persulfide-loaded cysteine desulfurase) that cleaves the acyl-adenylate and transfers sulfur thus forming the thiocarboxylate at the C-terminus of the small subunit (further details for the bacterial sulfur transfer are given by Leimkühler [16]).

In plants and humans, the MPT-synthase is activated by a sulfurase, a multi-domain enzyme, which is much larger than MoeB with which it shares one domain. The N-terminal domain is homologous to the bacterial MPT-synthase adenyl transferase MoeB,

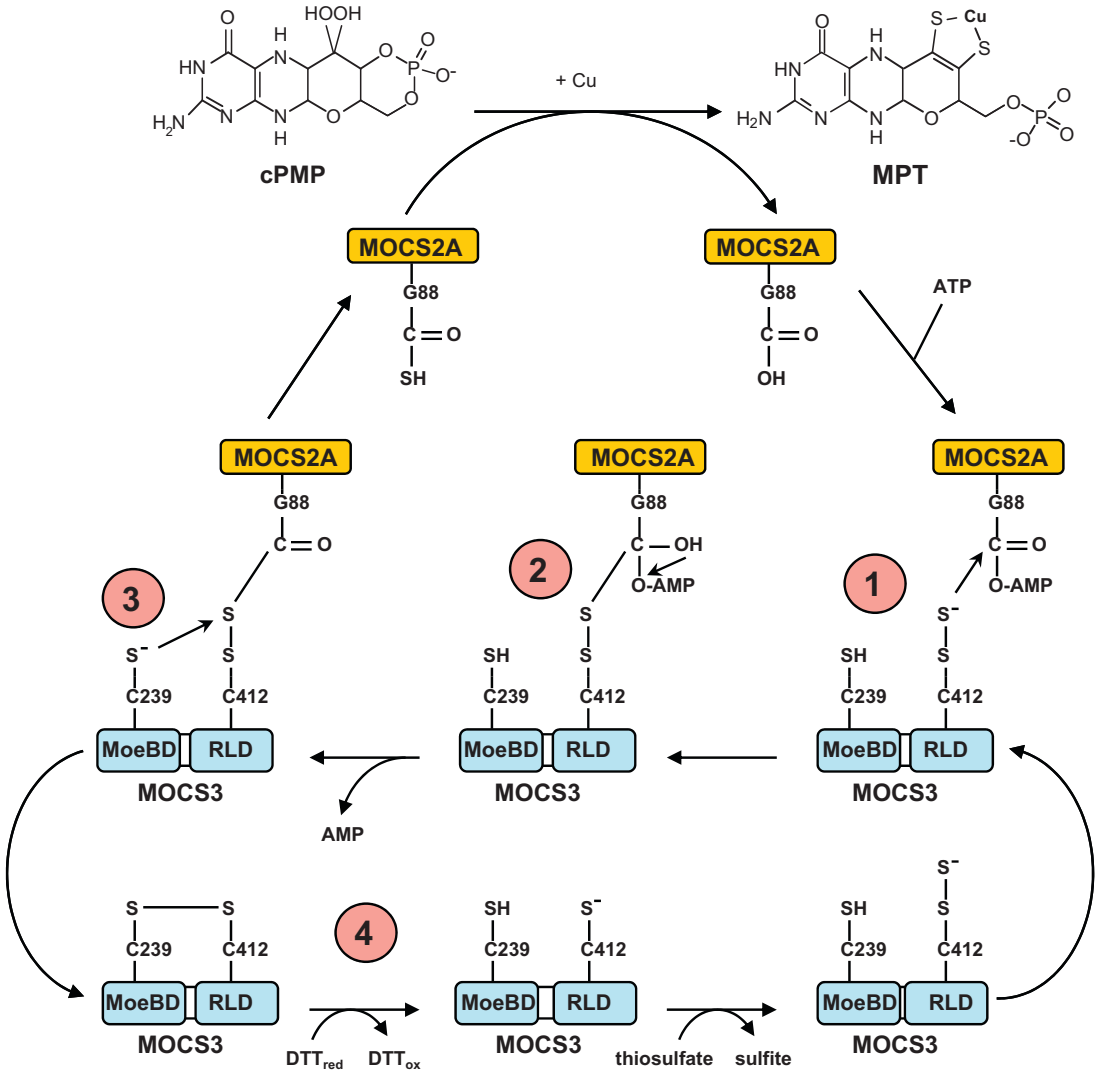


Fig. 4. Proposed mechanism for the sulfur transfer-mechanism from MOCS3 to the small subunit of MTP-synthase (MOCS2A). (1) MOCS3 carries a persulfide group at Cys412 on its rhodanese-like domain (RLD) that could serve as a nucleophile for the carboxylate at the C-terminus (Gly88) of MOCS2A, thus forming a disulfide intermediate (stage 2). (3) Reductive cleavage of the disulfide bond by attack of the thiol of Cys239 to form a disulfide bond with Cys412, in turn thiocarboxylated MOCS2A is formed. (4) The disulfide bond could be reduced in vivo by a thioredoxin system (in vitro by dithiothreitol). The role of copper will be explained in Section 4.6. MoeBD, MoeB-like domain. Modified after [49]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

while the C-terminal part forms a rhodanese-like domain (RLD) which is shown in Fig. 4. In this figure, a model is described that was suggested to explain the functions of the two domains [49]. Rhodaneses are defined as enzymes that catalyze in vitro the transfer of a sulfane sulfur from thiosulfate to cyanide while their biological role is as yet unknown. They bind the sulfur atom as persulfide to a highly conserved cysteine residue, which is also conserved in the RLD of Cnx5 and MOCS3 [56]. MOCS3-RLD binds the sulfur as persulfide and is able to thiocarboxylate the small subunit of MPT-synthase in vitro [49,56].

Therefore MOCS3 can be seen as a multi-functional protein combining the adenylation reaction (carried out by its N-terminal domain) with the subsequent sulfur transfer reaction (carried out by its C-terminal RLD) representing a nice example of product–substrate channeling during the evolution of this complex biosynthetic pathway. The identity of the donor for the reactive mobile sulfur is as yet unknown, but a redundant function of different persulfide-generating systems like cysteine desulfurases is possible. Surprisingly, there are mechanistic parallels between the functioning of MOCS3 and the activation of ubiquitin in higher organisms [57].

4.6. Step 3: adenylation of MPT

After synthesis of the MPT moiety, the chemical backbone is built to bind and coordinate the Mo atom (Fig. 2). In the following step, therefore, Mo has to be transferred to MPT in order to form Moco, thus linking molybdate uptake to the MPT pathway. This mechanism has been first uncovered in plants where the protein Cnx1 was found to catalyze a complex sequence of reactions [58,59] and where biochemical and structural studies revealed a novel biosynthetic intermediate, adenylated MPT (MPT–AMP) [60]. Consequently, the metal insertion reaction has to be subdivided into two separate steps, starting with the adenylation of MPT (Fig. 2).

The plant protein Cnx1 is a two-domain protein with homologies to two separate bacterial proteins [61]: the Cnx1G domain is homologous to the *E. coli* protein MogA und the Cnx1E domain to *E. coli* MoeA. Both bacterial proteins are involved in Mo insertion into MPT (for details see Leimkühler [16]), thus the modular nature of Cnx1 points to a functional cooperation between its two domains. Also the human counterpart to Cnx1, namely Gephyrin, is a two-domain protein of similar composition and shows similar activities [25,62]. It is interesting to note, that Gephyrin belongs

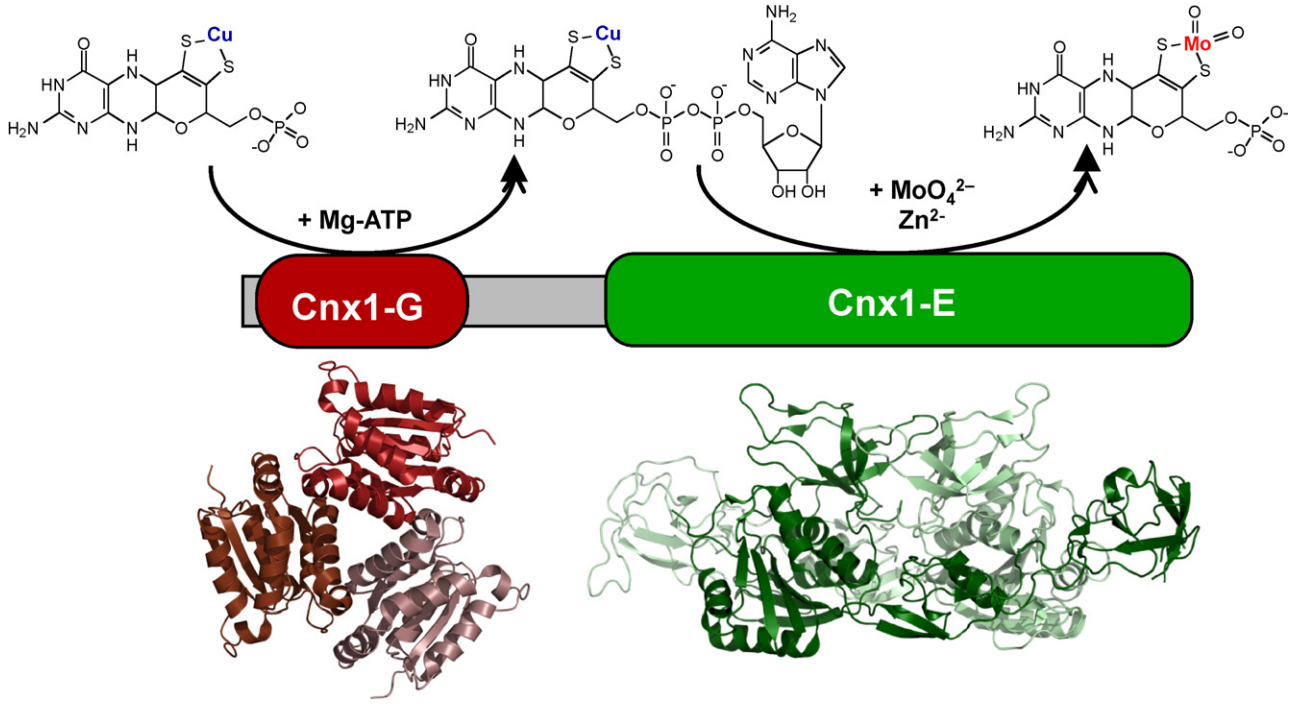


Fig. 5. Adenylation of MPT and metal insertion. The C-terminal G-domain of Cnx1 catalyzes MPT-adenylation using Mg–ATP, and the product MPT–AMP is transferred to Cnx1-E where MPT–AMP is hydrolyzed in a Mo-dependent manner, thus yielding Moco. Zinc ions promote the latter reaction. Additional products of the Cnx1 reaction are pyrophosphate, AMP and copper. The structures show the G-domain of Cnx1 [58] and the E-domain of Gephyrin [90].

to the family of *moonlighting* proteins as in the central nervous system it functions as scaffolding protein that binds and organizes inhibitory neuroreceptors [63].

Cnx1G was demonstrated to bind MPT, and the analysis of mutational variants of Cnx1G indicated separate protein regions for binding of MPT and for catalysis [58,59,64] although the exact reaction catalyzed by Cnx1G remained unclear. Only the crystal structure of Cnx1G purified with its reaction product (bound to a gain-of-function variant) led to the identification of MPT–AMP as novel reaction intermediate. In this structure, MPT was covalently bound via a pyrophosphate bond to an adenosine. Subsequently, the Mg–ATP-dependent synthesis of MPT–AMP was demonstrated for Cnx1G in vitro, and lack-of-function variants previously identified were unable to synthesize MPT–AMP [65].

The finding that MPT–AMP represents a general reaction intermediate in Moco biosynthesis was further extended by recent studies investigating the functional properties of Cnx1G-homologous proteins from *E. coli* (MogA) and the thermophilic bacterium *Pyrococcus furiosus* (MoaB), which both catalyze MPT–AMP synthesis [66].

During the structural analyses of Cnx1G in complex with MPT or MPT–AMP, another unexpected observation was the identification of copper bound to the MPT dithiolate sulfur atoms [60], whose nature was confirmed by anomalous scattering of the metal (Fig. 5). These structures show tetragonal coordination of copper suggesting a type I copper binding site for Cu^{1+}, which is also supported by EPR studies (Hille and Schwarz, unpublished results). Up to now the function of copper as MPT ligand is unknown but it might play a role in sulfur transfer to cPMP, in protecting the MPT dithiolate from oxidation, and/or presenting a suitable leaving group for Mo insertion. The origin of copper is also unclear but it is reasonable to assume that it is transferred in vivo via the action of cytoplasmic chaperones. It this context it is important to mention that in *E. coli*, where Cnx1G was expressed and purified, cytoplasmic copper is very low and therefore the observed copper binding to MPT seems to be very specific.

4.7. Step 4: Mo insertion

With the identification of MPT–AMP as intermediate in Moco biosynthesis, the question arose how MPT–AMP is converted into Moco and what function the other domain of Cnx1 might have. It turned out that MPT–AMP is transferred from Cnx1G to the Cnx1E-domain that cleaves the adenylate from MPT, releases copper and inserts Mo, thus yielding active Moco. It was found that Zn^{2+} hydrolyses MPT–AMP much faster than Mg^{2+}, but given the high abundance it is most likely that Mg^{2+} will be the major physiological co-substrate. The MPT adenylate was hydrolyzed in a molybdate-dependent way [66], and adenylated molybdate might occur as hypothetical reaction intermediate as depicted in Fig. 6. This reaction is coupled to the metal exchange reaction where bound copper is released and Mo is transferred to the MPT dithiolate, resulting in Moco release. Moco formed by Cnx1E most probably carries two oxo ligands and one OH-group in a deprotonated form [66], as supported by preliminary spectroscopic data derived from a storage-protein-bound Moco (see below; George and Schwarz, unpublished data). There is no experimental evidence for a reduction of Mo at this stage.

Plant mutants defective in Cnx1 and mammalian mutants defective in its homolog Gephyrin show a molybdate-repairable phenotype, i.e., Moco biosynthesis can be restored at least partially by growing the mutant cells on media containing unphysiologically high concentrations (1–10 mM) of molybdate [67,68]. These extreme conditions override the effect of the mutation in Cnx1 while physiological Mo concentrations are 1000 fold lower and need catalysis by functional Cnx1 to effectively insert Mo into MPT.

In comparison to bacteria, higher organisms coupled the two consecutive steps leading to Mo insertion into single proteins with two domains. Both domains were fused at least two times during evolution resulting in multi-domain proteins with different orientations of the G and E domains (plants have the E domain on the N-terminus of the protein, in mammals and fungi it is the G domain). These evolutionary distinct events point to the high

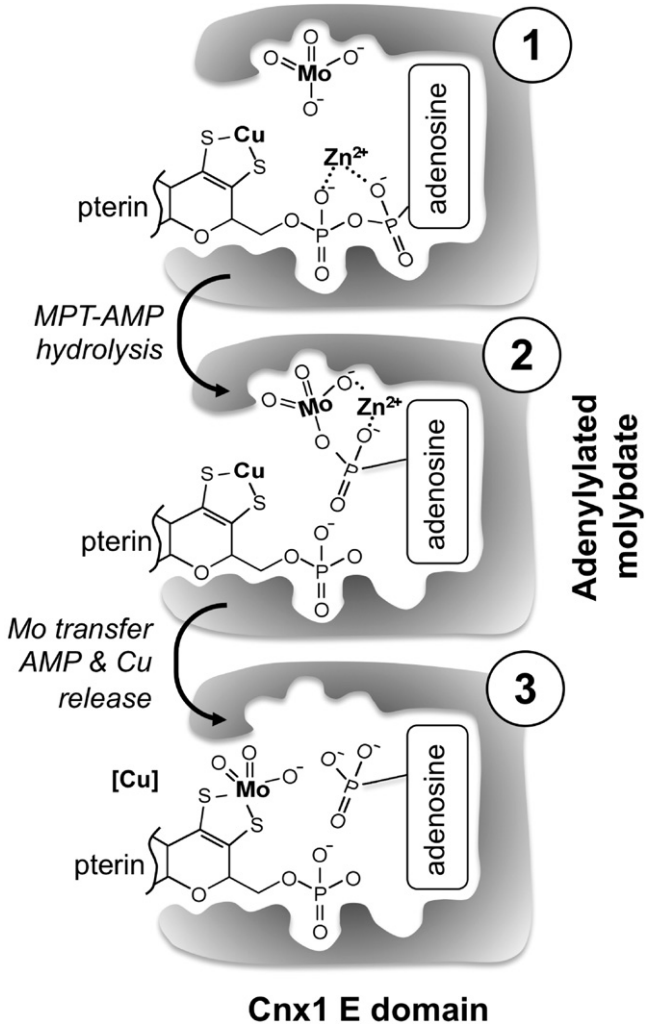


Fig. 6. Proposed mechanism for molybdate activation and transfer to MPT. (1) Adenylated MPT (MPT–AMP) and molybdate bind first in a cooperative manner to the Cnx1-E domain. (2) Formation of a hypothetical reaction intermediate (adenylated molybdate), which is thought to represent an unstable transition state that will immediately react to MPT, thus replacing bound copper at the MPT dithiolate (stage 3). The function of copper is still unknown, and it remains unclear whether Mo insertion is dependent on copper.

pressure and functional benefit of having the adenylation function and the metal insertion function coupled into one protein thus providing an excellent product–substrate channeling. As a result of different domain orientations, the resulting linking-peptides evolved differently and therefore it is not surprising that in the human protein Gephyrin a so-called central domain developed harboring binding sites for a number of different proteins. These interaction partners are required for Gephyrin's 'second' function, as it is serves a structural role in binding and anchoring inhibitory neuroreceptors (ligand-gated anion channels) in the postsynaptic membrane of neurons (i.e., the signal receiving nerve cell). It is remarkable to see how basic metabolic pathways served as a tool-box for novel functions in special tissues of higher organisms.

5. Storage and transfer of Moco

For most prosthetic groups there is a gap of knowledge in our understanding how after completion of biosynthesis the prosthetic groups are directed to their various cellular destinations and how they ultimately find the way into their correct cognate proteins, or whether they are stored after synthesis. Intricate mechanisms can be assumed to control distribution, trafficking, and insertion into proteins as most of these prosthetic groups are extremely 'fragile' and air-sensitive. Thus it was no surprise when it turned out that – beyond biosynthesis – there exists no free pool of prosthetic groups in the cell. Consequently, there must be a plethora of transporters, binding proteins, storage proteins, chaperones and insertases that ensures safe transport, protection and insertion of prosthetic groups, however this complex machinery is largely unexplored.

For Moco in higher organisms, some pieces of such a sorting machinery became known in recent years. After completion of biosynthesis, Moco has to be allocated and inserted into the apo-Mo-enzymes, or Moco has to become bound to a carrier protein that protects and stores Moco until further use thus providing a way to buffer supply and demand of Moco. In bacteria, a complex of proteins synthesizing the last steps of Moco biosynthesis donates the mature cofactor to apo-enzymes assisted by enzyme-specific chaperones (details are given in the reviews by Leimkühler [16] and by Weiner [17]). Chaperones are proteins helping other proteins to fold and they also assist proteins to correctly insert prosthetic groups. In bacteria, nearly each Mo enzyme has a private chaperone available, however in higher organisms no Mo-enzyme specific chaperones have been found yet. As free Moco is extremely sensitive to oxidation, it is also assumed that Moco occurs permanently protein-bound in the cell. Therefore, a cellular Moco distribution system should meet two demands: (i) it should bind Moco subsequent to its synthesis, and (ii) it should maintain a directed flow of Moco from the Moco donor Cnx1-E to the Moco-dependent enzymes. This is important in order to ensure the fast and efficient incorporation of the cofactor into apo-Mo-enzymes.

In the green alga *Chlamydomonas reinhardtii* a Moco carrier protein (MCP) was identified which was found to tightly bind Moco thus protecting it against oxidation [69,70]. Without any denaturing procedure, subsequent transfer of Moco from MCP to apo-nitrate reductase from *Neurospora crassa* was possible [71]. These properties of *Chlamydomonas* MCP make it a promising candidate for being part of a cellular Moco delivery system. It is however unknown whether MCP is also able to donate Moco to Mo-enzymes other than nitrate reductase. The crystal structure shows that *Chlamydomonas* MCP (Fig. 7A) is a homotetramer with each 16 kDa monomer binding one molecule of Moco and possessing a typical Rossmann fold [71]. Preliminary data suggest that Mo is bound in a tri-oxo coordinated form in MCP (George and Schwarz, unpublished results). Structure-guided mutagenesis has identified a surface depression that might function in Moco binding [71], however, a complex structure of MCP with Moco is still missing.

In the higher plant *Arabidopsis thaliana*, recently a novel protein family was identified consisting of eight members that all can bind Moco and are therefore named Moco-binding proteins (MoBP) [72]. These proteins form 50 kDa dimers, bind Moco less tightly than *Chlamydomonas* MCP and are therefore no good candidates to serve as Moco storage proteins. Rather they seem to be involved in the cellular distribution of Moco because in the cytoplasm of a living cell they were found to undergo protein–protein interaction both with the 'Moco-donor' protein Cnx1 and the 'Moco-user' protein nitrate reductase [72]. Consequently, they were also able to deliver Moco to apo-nitrate reductase. Although they share only 20% primary sequence identity with *Chlamydomonas* MCP, their crystal structure (Fig. 7B) shows a high degree of structural conservation to *Chlamydomonas* MCP. However, in contrast to MCP from algae, plant MoBP proteins bind Moco in a different mode as their flexible C-terminus was shown to be essential for Moco-binding [72]. This flexible C-terminus is lacking in *Chlamydomonas* MCP. The high number of eight expressed MoBP proteins in *Arabidopsis* probably points towards an organ and tissue specific functional specialization.

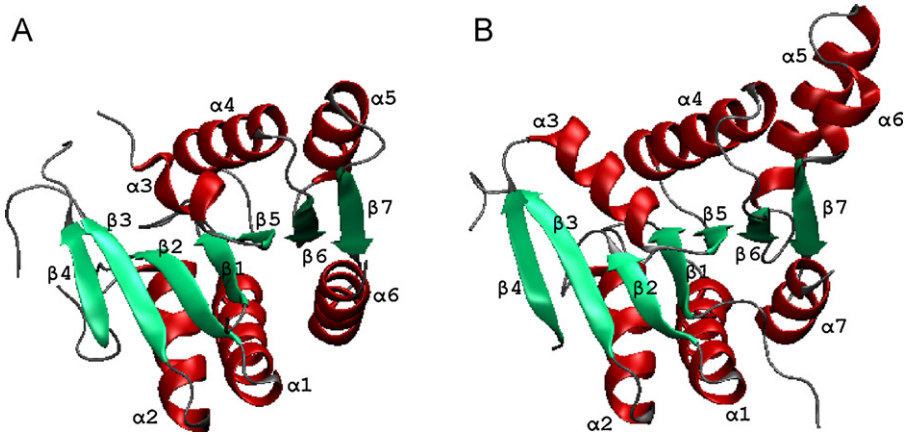


Fig. 7. Atomic structures of Moco carrier and binding proteins in ribbon representations. (A) Moco carrier protein MCD from the alga *Chlamydomonas rheinhardtii* [71]. (B) Moco binding protein MoBP3 from the higher plant *Arabiodpsis thaliana* [72].

6. Insertion of Moco into Mo-enzymes and final maturation

Insertion of Moco into Mo-enzymes is still not understood, however there are ideas how insertion could take place (compare the review by Hille [3]). All crystal structures of Mo-enzymes demonstrated that the cofactor is deeply buried within the holo-enzyme [13,73] so that Moco might have been incorporated only prior to or during completion of folding and dimerization of the apoprotein monomers. Using a defined in vitro-system it was shown that human apo-sulfite oxidase can directly incorporate Moco [74]. However, for insertion of Moco into the target apo-enzymes as it occurs in the living cell either (still unknown) chaperone proteins would be needed, or the Moco carrier/binding proteins could become involved at this stage.

As there are three classes of Mo enzymes known in higher organisms (Hille [3]) also differences in the insertion of Moco might be considered. Fig. 8 shows a scheme where we have summarized Moco-biosynthesis and its allocation to these three classes of user-enzymes. Enzymes of the sulfite oxidase family are characterized by a highly conserved cysteine residue providing a third sulfur atom in the Mo center. In mature Moco, the proposed tri-oxo coordination of Mo (compare Fig. 6) suggests a simple mechanism of cysteine ligation to the Mo atom accompanied by loss of one of the oxygen atoms as water. One could assume that the Moco-binding MoBP proteins described above could participate in this process. They are not only involved in the cellular distribution of Moco but were also shown to facilitate insertion of Moco into apo-nitrate reductase [72]. Preliminary experiments indicate that they do not interact with xanthine dehydrogenase in plants (Mendel and unpublished observation). Therefore one could cautiously regard these proteins as "private Moco chaperones" specific for members of the sulfite oxidase family but further experiments are needed to support this assumption. This assumption does not exclude a direct transfer of Moco from the donor protein Cnx1-E to the Mo-enzyme, which has been shown in vitro [66].

In contrast, enzymes of the xanthine oxidoreductase family contain a terminal sulfido group as third ligand in their active center. To gain enzymatic activity, in xanthine oxidoreductases and aldehyde oxidase this sulfur atom is added to the enzyme protein in a final step of maturation, catalyzed by the enzyme Moco sulfurase (ABA3 in plants, HMCS in humans; Fig. 8). Mechanistic details of this step are given in the review by Hille [3]. Preliminary data indicate that Moco sulfurase physically interacts with the Moco donor protein Cnx1-E (Mendel, unpublished observation) and likewise is able to form a complex with the acceptor enzyme xanthine dehydrogenase [75]. Therefore one could cautiously regard these proteins as "private chaperone for sulfurated Moco" specific for members of the xanthine oxidoreductase family. But this route is obviously not the only way to supply Moco to Mo-enzymes of this family because in mutants lacking Moco sulfurase the enzymes xanthine dehydrogenase and aldehyde oxidase contain Moco, that however is not sulfurated [75]. This finding can only be explained by a direct transfer of Moco from the donor protein Cnx1-E to the Mo-enzyme. Hence, the task of a Moco sulfurase would be to exchange non-sulfurated for sulfurated Moco thus activating the enzyme. More detailed explanations are discussed by Hille [3].

The novel Mo-enzyme mitochondrial amidoxime reductase mARC is suggested to form a new class of Mo-enzymes in higher organism [76]. Neither it has a terminal sulfido group as third ligand in the active center, nor it shows an active center similar to the sulfite oxidase family [3]. It will be interesting to see whether the Moco-binding MoBP proteins will be able to interact with this enzyme.

7. Molybdenum–iron and molybdenum–copper homeostasis

Iron plays an important role for the synthesis of Moco as Cnx2 (human MOCS1A), which is involved in step 1 of Moco biosynthesis, requires two Fe–S clusters of the [4Fe–4S] type (Fig. 8). These clusters are essentially involved in the SAM-dependent mechanism of cPMP formation [41] and have to be preassembled in the mitochondria, where mitochondrial as well as extramitochondrial (Fe–S) clusters originate from [77].

Another crosslink between iron and Mo has been found subsequent to step1 of Moco biosynthesis. As outlined above, the transporter protein Atm3 is associated with the export of cPMP from the mitochondria into the cytosol [46] (Fig. 8). This transporter has obviously a dual function: it not only exports cPMP but also an as yet unidentified precursor for Fe–S cluster synthesis in the cytosol [51]. Therefore mutants in the Atm3 exporter become short of both cPMP and Fe–S clusters in the cytosol with the consequence that all enzymes needing these prosthetic groups decrease in activity.

Beyond Moco biosynthesis and allocation there is a third link between Mo and iron metabolism. Both Mo-enzymes xanthine dehydrogenase and aldehyde oxidase depend on iron in the form of [2Fe–2S] clusters, whereas nitrate reductase requires iron in the form of iron–heme (Fig. 8). Summarizing the Mo–iron crosstalk it becomes evident that Moco biosynthesis and the functioning of the majority of Mo-enzymes in higher organisms is strictly dependent on iron metabolism for providing Fe–S clusters and iron–heme groups. Any impairment of iron metabolism would immediately affect Mo metabolism as well.

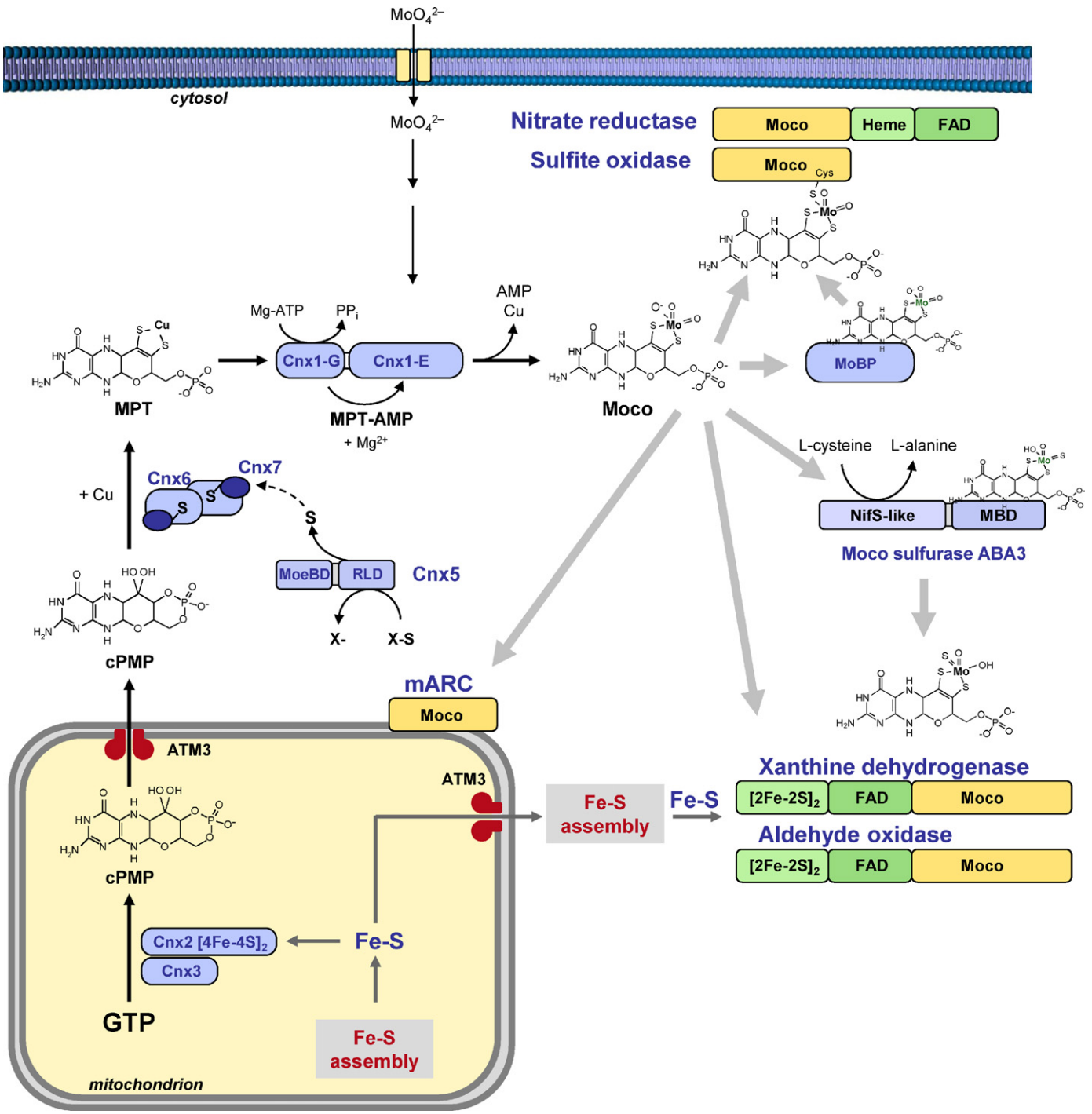
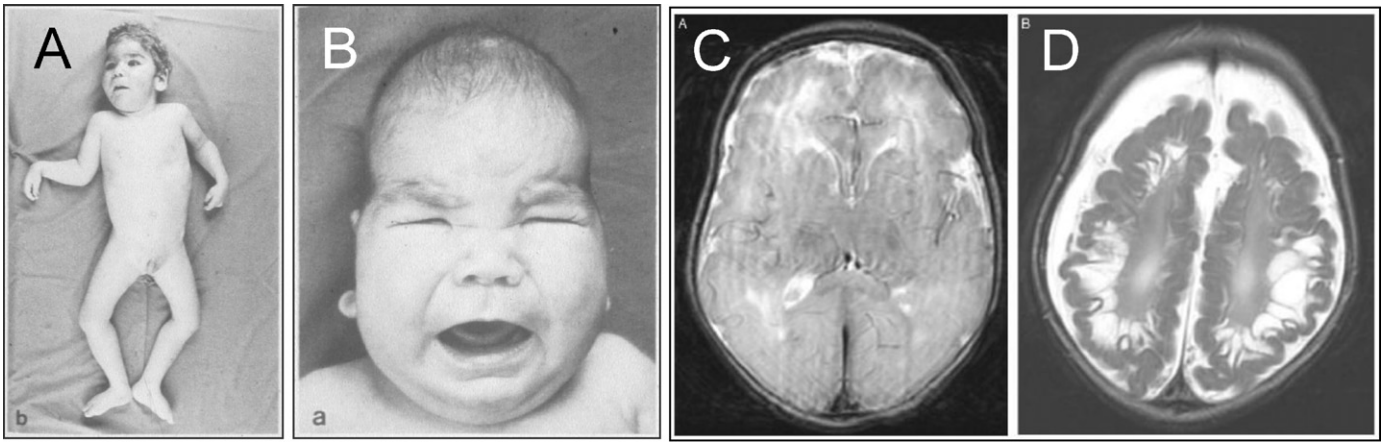


Fig. 8. Moco biosynthesis and allocation in higher organisms (plants). Moco biosynthesis starts with the conversion of GTP to cPMP in the mitochondria, all subsequent steps proceed in the cytosol. Moco biosynthesis enzymes and Moco-binding proteins are shown in blue. The dependence of Cnx2 on Fe–S and SAM is indicated. MPT-synthase, consisting of Cnx6 and Cnx7, is sulfurated by Cnx5, with the primary sulfur donor (X-S) mobilized by the rhodanese-like domain of Cnx5 (RLD) being unknown. The adenylation domain of Cnx5 (MoeBD) is required for adenylation and activation of the small MPT synthase subunit Cnx7. It is assumed that copper (Cu) is inserted directly after dithiolene formation. The individual reactions of Cnx1 and its products (Moco, pyrophosphate PP_i, AMP, copper) are indicated. Mature Moco can be either bound to a Moco-binding protein (MoBP), to the Mo-enyzmes mARC, nitrate reductase, sulfite oxidase, xanthine dehydrogenase and aldehyde oxidase, or to the Moco-binding C-terminal domain of the Moco sulfurase ABA3 protein (MBD). ABA3 generates a protein-bound persulfide, which is the source of the terminal sulfur ligand of Moco in xanthine dehydrogenase and aldehyde oxidase. Like Cnx2, xanthine dehydrogenase and aldehyde oxidase depend on Fe–S-clusters, which are synthesized in mitochondria prior to export by transporter ATM3 and assembly in the cytosol.

Copper has been found to be associated with MPT and MPT–AMP [60]. In early reports, Mo was found to act antagonistically to copper. The shortage of molybdate in Australian farmland triggered excessive fertilization, resulting in molybdate overload of the soil that caused pathologic symptoms of molybdenosis in animals, which in particular in ruminants triggered secondary copper-deficiency [78]. Later, these Mo-induced conditions of copper-deficiency revealed the pathology of two human Cu-homeostasis disorders: Menkes (Cu-deficiency) and Wilson's (Cu-overload) diseases [79]. Consequently, potent Cu-chelators such as tetrathiomolybdates were used to treat Wilson's disease and a number of other disorders that are linked to Cu-homeostasis,



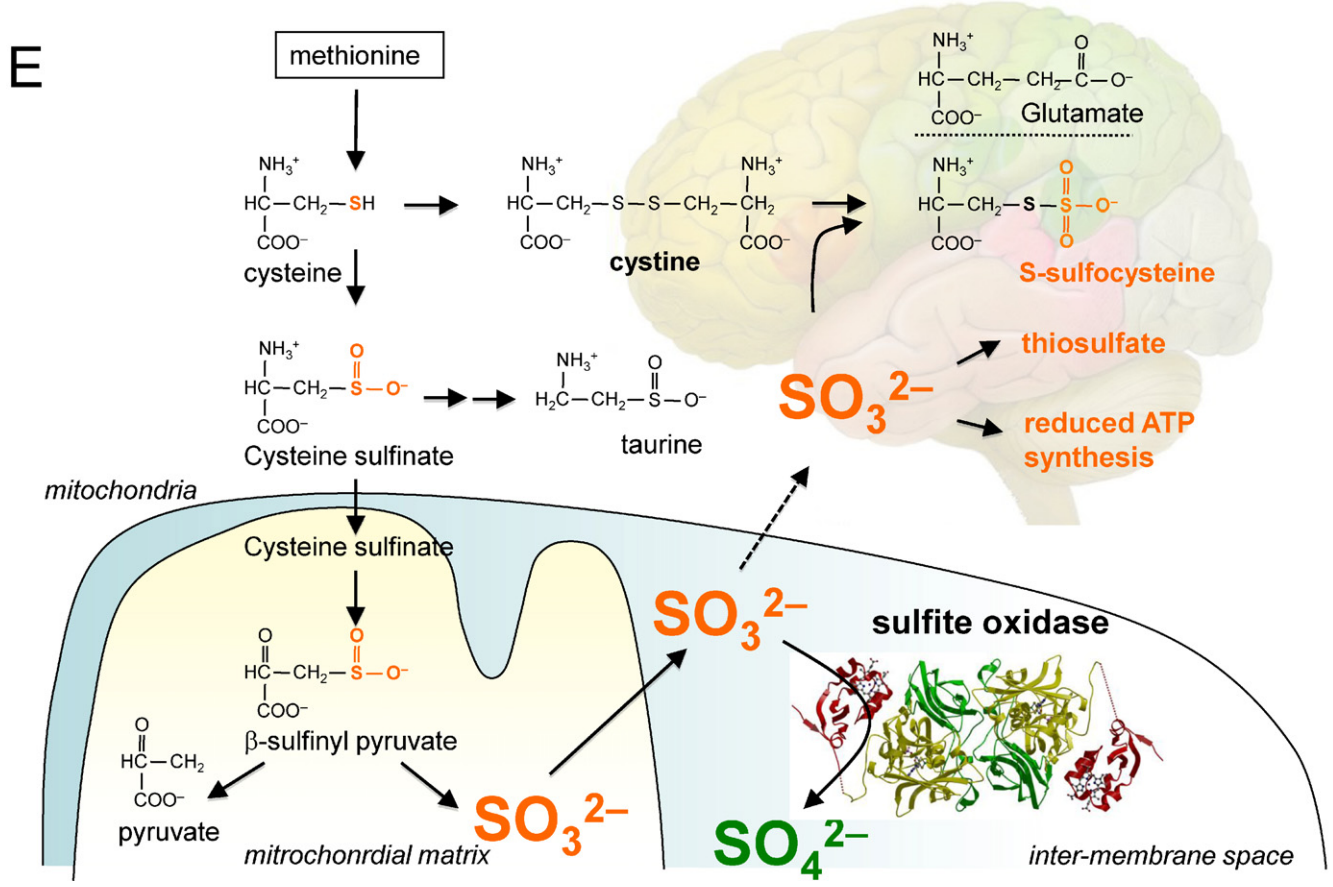


Fig. 9. Patients with Moco deficiency. Increased tonus (spasm) (A) and dismorphic head due to progressive microcephaly (B) in the age of 22 (A) and 3.5 months (B) [91]. Magnetic resonance images at 11 days (C) and 3.5 months (D) of age show progressive brain atrophy and changes in the subcortical white matter [84]. (E) Cysteine catabolism starts in the cytosol and proceeds in the mitochondrion where highly reactive sulfite is generated that becomes detoxified by sulfite oxidase. In patients suffering from Moco deficiency, sulfite and taurine accumulate, while cystine is depleted due to its reaction with sulfite, the latter is resulting in the formation of S-sulfocysteine.

such as neurodegeneration, cancer and inflammation [80]. The underlying chemistry of the chelation of either copper or Mo to dithiolates most likely explains their antagonistic function towards each other and future studies will show to which extend alterations in copper homeostasis might contribute to Mo enzyme activities.

In vitro studies with Cnx1-G-bound MPT–AMP revealed an inhibition of Moco synthesis in the presence of 1 μM $CuCl_2$, providing a link between Mo and copper metabolism [60]. Copper inhibition of Moco synthesis can be explained by inhibition of the Mg-dependent Mo insertion reaction. The latter is supported by the suppression of copper inhibition with equimolar amounts of Cnx1-E and is in line with the known copper inhibition of pyrophosphatases [81]. This finding might suggest that Moco biosynthesis might be affected under conditions when cellular copper concentrations are increased, as seen in human patients affected with Wilson's disease [79], where copper accumulates in liver and brain, resulting in severe damage to both organs. But also copper shortage might affect Moco biosynthesis, which remains to be elucidated and should shed light onto the question what function copper might serve in Moco synthesis.

8. Human molybdenum cofactor deficiency and therapy

Human Moco deficiency resides in a loss of activity of all Mo-dependent enzymes such as sulfite oxidase, xanthine oxidase and aldehyde oxidase [20]. Moco deficiency is a hereditary recessive disorder that affects neonates soon after birth. While patients are born normally they develop intractable seizures within days after birth and require intensive care and anticonvulsant treatment. As seen in other inborn errors of metabolism (such as urea cycle disorders), symptoms develop shortly after birth, when the babies' metabolism starts to operate and toxic metabolites are building up within the body. In Moco deficiency, the major cause of the disease resides in the loss of sulfite oxidase, which effectively removes sulfite from the human body. A loss in sulfite oxidase activity results in the build up of sulfite, ultimately causing severe neurological damage, disordered autonomic function, exaggerated startle reactions, dysmorphic facial features, alterations in muscle tone, progressive cerebral palsy, microencephaly, seizures and death (Fig. 9). Patients that survive the acute initial phase of seizures, survive only few years with basically no neuronal development. Patients are unable to make any coordinated movements, need to be tube fed and show no signs of communication with their environment. The incidence of Moco deficiency is below 1:100,000, however, many missed, non-diagnosed and non-reported cases are suspected and therefore prevalence determination is limited. Due to the lack of any causative treatment, death in early childhood has been the usual outcome.

The major cause of brain damage in Moco deficiency is sulfite accumulation. Sulfite is formed as intermediate product of the cysteine degradation pathway, which serves as the main sulfur exit for the catabolism of sulfur-containing amino acids and sulfo-lipids. Cysteine catabolism (Fig. 9) starts with the oxygen transfer yielding cysteine sulfinate, the precursor of taurin. Subsequently, cysteine sulfinate is translocated into the mitochondrial matrix, where an aminotransferase synthesizes b-sulfinyl pyruvate, an unstable molecule that spontaneously decomposes into pyruvate and sulfite. The latter "leaks" out into the mitochondrial inter-membrane space, where the Moco-dependent sulfite oxidase is localized that converts sulfite into sulfate. The oxidation is coupled to the reduction of cytochrome *c*, the terminal electron acceptor of the reaction. Sulfite oxidase has been studied intensively, the reaction mechanism is well understood and its crystal structure was found to be the prototype of an entire enzyme family [13].

Under conditions of Moco- or isolated sulfite oxidase-deficiency, sulfite leaves the mitochondria and finally accumulates extracellularly in plasma, crosses the blood–brain-barrier and rapidly triggers neuronal death [83]. Sulfite is a highly reactive molecule, a strong reductant, which attacks disulfide bridges thereby affecting the function and integrity of extracellular proteins and transmembrane receptors. Furthermore, sulfite depletes cystine, the major carrier of cysteine in plasma and serum. In particular, the synthesis of glutathione, the main cellular antioxidant, is highly dependent on extracellular cystine. Finally, and probably most importantly, sulfite-mediated cystine depletion results in the formation of S-sulfocysteine, a metabolite, which is present at very low levels in healthy individuals [82]. S-sulfocysteine is structurally similar to glutamate and therefore believed to activate another group of neuroreceptors. The latter may explain the observed seizures, convulsions, contractions and twitching associated with Moco deficiency, which is further accompanied by abnormal magnetic resonance imaging and loss of white matter due to cortical damages [83]. As hyperexcitation is a well known determinant of neuronal death, it is not surprising to see that basic mitochondrial functions are affected upon sulfite exposure, such as the reduction in ATP synthesis [84].

In order to study Moco deficiency, a mouse animal model has been generated [85]. As molecular genetics of Moco deficiency patients revealed the highest prevalence for mutations in the *mocs1* gene [21] a *mocs1*-deficient knock out model was created and characterized [85]. Homozygous *mocs1* mice display a severe phenotype that reflects all biochemical characteristics of human Moco deficiency patients. They fail to thrive and die within the first 10 days of life with an average life span of 7.5 days. As *mocs1*-deficient mice are unable to synthesize cPMP, we developed a method to enrich, isolate and purify cPMP from *E. coli* for subsequent treatment approaches [33]. Purified cPMP was injected in different doses right after birth into the liver of *mocs1*-deficient and healthy mice [86]. As a function of the amount of cPMP, which was injected every second to third day, average survival time increased dramatically and treated mice developed normally, gained weight and reached adulthood and fertility like their wild-type littermates. Notably, withdrawal of cPMP from *mocs1*-knockout mice caused death within 10–14 days. Treated animals behaved normally, were indistinguishable from healthy littermates and were fertile.

Given to promising results in the animal model, we further improved the fermentation procedure for cPMP, in order to be able to have a process in place that allows sufficient bio-production of this unstable and reactive Moco intermediate. In 2008, a patient with Moco deficiency was born and diagnosed on day six of life [87]. The infant's urine revealed markedly elevated levels of S-sulfocysteine, thiosulfate and xanthine, low plasma and urine uric acid, elevated urine sulfite and undetectable cPMP, being indicative for a deficiency in MOCS1, which has been genetically confirmed later [87]. At day six, magnetic resonance imaging showed diffuse cerebral edema with an elevated lactate peak in the magnetic resonance spectroscopy. The infant had a markedly abnormal electro encephalogram at seven days of age.

Following intensive discussion with the Regulatory Authorities, the first patient has received cPMP treatment on day 36 of life. As starting dose, 80 μg cPMP per kg body weight were chosen based on previous studies in *mocs1*-deficient mice [88]. Beside sulfite excretion, we have used S-sulfocysteine as well as xanthine and uric acid (xanthine oxidase substrate and product) as biomarkers to monitor treatment efficacy. Within days after treatment was started, urinary markers of sulfite oxidase and xanthine oxidase deficiency returned to almost normal readings and stayed constant. Clinically, the patient became more alert a few days after the treatment started, the convulsions and twitching disappeared within the first two weeks as documented by an electro encephalogram showing the return of rhythmic elements and markedly reduced epileptiform discharges. Based on the first index case, a treatment plan was developed for a cPMP therapy of Moco deficiency patients and in the meantime, four other Moco deficiency patients have received cPMP treatment and show the same biochemical and clinical improvement as the first patient (Schwarz et al., unpublished data).

9. Conclusion and outlook

Now that the basic principles of Moco biosynthesis are understood, future research will focus on the mechanistic details of the single reactions. For example the reaction product of Cnx2/MOCS1A needs to be identified and the subsequent reaction leading to cPMP has to be uncovered. The mechanism of MPT synthesis is still not completely understood and the requirement of copper in Moco biosynthesis is important to understand.

In higher organisms, the steps beyond Moco biosynthesis are less well known and, therefore, research will also focus on Moco transport, allocation and insertion into apo-enzymes. Here one has to keep the cellular context in mind as Moco biosynthesis occurs to be micro-compartmentalized in a multiprotein-biosynthesis com-

plex localized in the cytosol of the cell. The systems of molybdate uptake into the cell are far from being clear in higher organisms and need further research. In addition the maturation of enzymes such as the animal sulfite oxidase, which needs to be translocated into the mitochondrial inner membrane space needs to be investigated as it either requires an independent transport of Moco into mitochondria or a novel mechanism of translocating sulfite oxidase together with Moco in (at least) a semi-folded state, for which we have recently obtained preliminary data (Schwarz, unpublished results).

In addition, the role of other homeostatic circuits, such as copper and iron metabolism, awaits further investigation, which could address questions regarding the pathophysiology of related metabolic disorders. Finally, chemical synthesis of Moco and respective intermediates are still a challenging task, and, once achieved, very basic questions regarding the contribution of the pterin in catalysis and/or electron transfer can be addressed. A main focus should be the synthesis of cPMP for which an immediate application in treating MoCD patient would arise.

Acknowledgements

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