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Current Opinion in Chemical Biology

Molybdenum cofactor and human disease

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Four molybdenum-dependent enzymes are known in humans, each harboring a pterin-based molybdenum cofactor (Moco) in the active site. They catalyze redox reactions using water as oxygen acceptor or donator. Moco is synthesized by a conserved biosynthetic pathway. Moco deficiency results in a severe inborn error of metabolism causing often early childhood death. Disease-causing symptoms mainly go back to the lack of sulfite oxidase (SO) activity, an enzyme in cysteine catabolism. Besides their name-giving functions, Mo-enzymes have been recognized to catalyze novel reactions, including the reduction of nitrite to nitric oxide. In this review we cover the biosynthesis of Moco, key features of Moco-enzymes and focus on their deficiency. Underlying disease mechanisms as well as treatment options will be discussed.

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Current Opinion in Chemical Biology 2016, **31**:179–187

This review comes from a themed issue on **Bioinorganic chemistry**

Edited by **R David Britt** and **Emma Raven**

For a complete overview see the Issue and the Editorial

Available online 4th April 2016

http://dx.doi.org/10.1016/j.cbpa.2016.03.016

Introduction

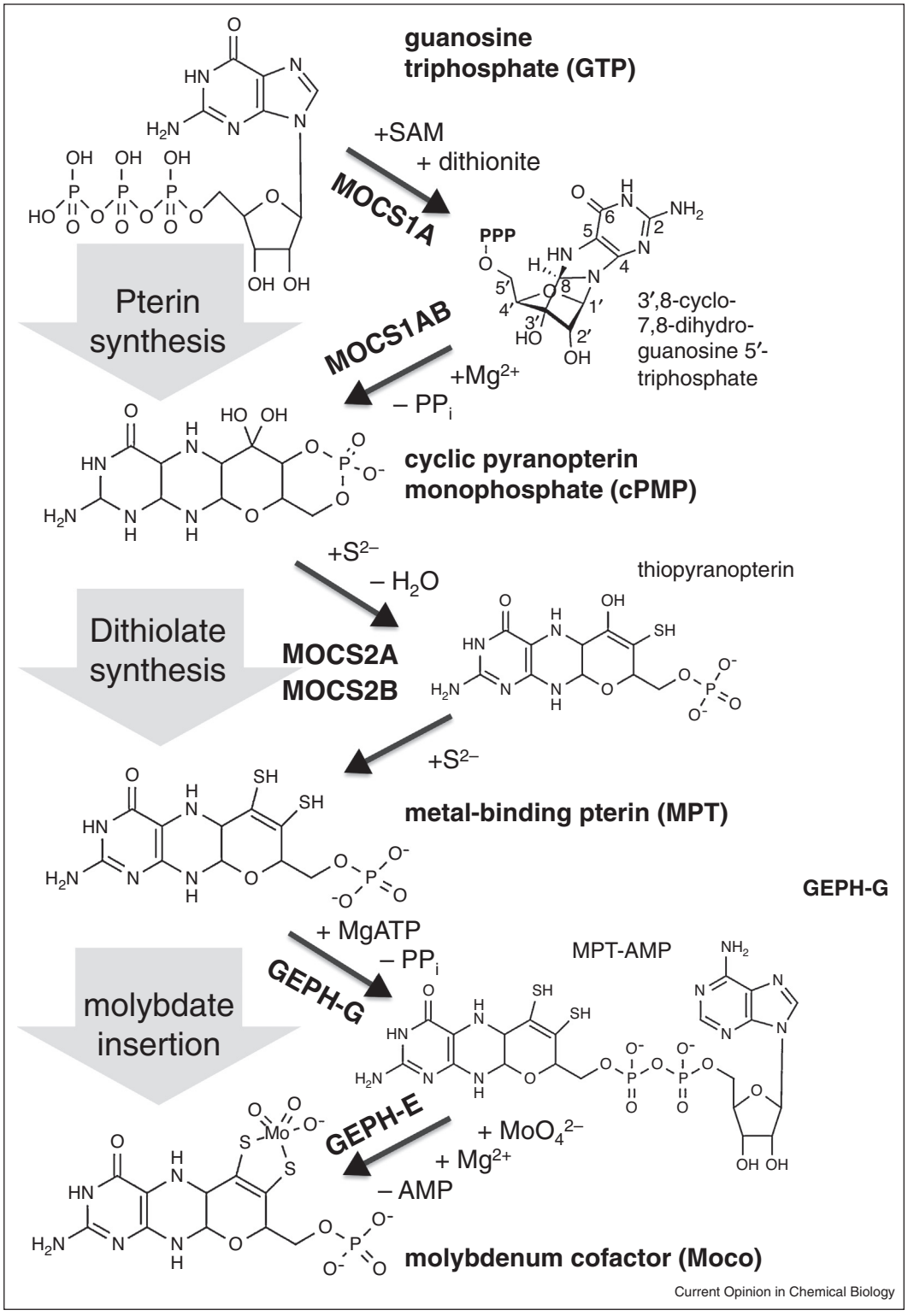
Molybdenum (Mo) is the only trace metal of the second row of the periodic table that exhibits biological activity when it is ligated to a cofactor. In nature two principal concepts of Mo cofactors have evolved, one is the iron Mo cofactor in bacterial nitrogenase and the other is represented by a large family of enzymes with more than 100 representatives relying on the pterin-based Mo cofactor (Moco) [1]. Moco-containing enzymes catalyze key redox reactions in the global carbon, sulfur and nitrogen cycles [2•]. The overall reaction is characterized by the transfer of an oxygen atom to or from a substrate in a two-electron transfer reaction [2•]. Moco consists of a Mo atom covalently bound via the dithiolate moiety of a fully reduced pterin backbone with a pterin C6-substituted four-carbon side chain forming a third pyran ring, commonly referred to as molybdopterin (MPT) or metal binding pterin [3] (Figure 1). Moco is found in all kingdoms of life, with most representatives in Prokarya that chelate Mo by either one or two MPT moieties harboring additional modification by guanine or cytosine [4]. In the following, we focus on the function of Moco in men; however, many aspects can be generalized to eukaryotic Moco synthesis.

Molybdenum cofactor biosynthesis

In all kingdoms of life, Moco is synthesized by a conserved biosynthetic pathway that can be divided into four steps [3], according to the biosynthetic intermediates cyclic pyranopterin monophosphate (cPMP), MPT and adenylated MPT (MPT-AMP) (Figure 1). Moco biosynthesis starts with the conversion of GTP into cPMP in a complex rearrangement reaction catalyzed in humans by two proteins, MOCS1A and MOCS1AB. Both proteins are expressed from the *MOCS1* gene encoding a variety of alternatively spliced transcripts [5] of which one variant (type I) expresses the MOCS1A protein encoded by exons 1–9. The transcript also contains a second open reading frame encoded by exon 10 [5]. Other variants (type II and III) represent transcripts that are derived from a truncation or deletion of exon 9 causing a loss of the first stop codon and a continuous open reading frame encoded by exon 1–10, representing the MOCS1AB protein with an additional MOCS1B domain [5]. MOCS1A binds two [4Fe-4S] clusters and belongs to the family of radical S-adenosylmethionine-dependent enzymes of the glycyl radical type [6]. The bacterial orthologues of MOCS1A and the MOCS1B domain, MoaA and MoaC, respectively, have been studied intensively leading to the discovery of their structure, reaction mechanisms, and unique reaction intermediates [7,8]. Recently, the complex reaction converting GTP into cPMP has been dissected thus demonstrating that the radical SAM protein MoaA converts GTP into 3′,8-cyclo-7,8-dihydro-GTP (Figure 1) and that this reaction is dependent on the C-terminal double glycine motif of MoaA and MOCS1A providing an essential mechanism to trigger the free radical reaction [9]. Subsequently, 3′,8-cyclo-7,8-dihydro-GTP undergoes major rearrangement reaction at the MoaC protein to yield cPMP with the pyran ring, a germinal diol and cyclic phosphate [10••,11]. The chemical structure of the first stable intermediate in Moco biosynthesis, cPMP (Figure 1), has been clarified by ^{13}C NMR studies [12]. Chemical synthesis of cPMP has been achieved recently, representing the first synthetic route for a biologically active derivative of Moco [13••].

In the second step of Moco biosynthesis, two sulfur atoms are transferred to cPMP to form the MPT dithiolate. The reaction is catalyzed by the enzyme MPT-synthase, a

Figure 1



Biosynthesis of the molybdenum cofactor. Major and transient intermediates of the three steps are shown. Cosubstrates for each reaction step are depicted at the arrows.

hetero-tetrameric complex of two small (MOCS2A) and two large (MOCS2B) subunits [4] (Figure 1). The small subunit carries the sulfur as C-terminal thiocarboxylate and represents the evolutionary ancestor of ubiquitin. Sulfur transfer to MOCS2A is dependent on MOCS3, a protein with an N-terminal MoeB-like domain, essential for the adenylylation of MOCS2A, and a C-terminal rhodanese domain catalyzing the sulfur transfer to MOCS2B [4]. Recently, it was shown that MOCS3 interacts with tRNA thiouridine modification protein (TUM1), also designated as 3-mercaptopyruvate sulfurtransferase, suggesting multiple roles of MOCS3 in sulfur transfer reactions [14].

The third and fourth steps of Moco biosynthesis represent two succeeding reactions, resulting in the adenylylation of MPT and subsequent molybdenum insertion [1], respectively. In humans, this reaction is catalyzed by the multifunctional protein gephyrin, consisting of an N-terminal G-domain (MPT adenylyltransferase, GEPH-G) and C-terminal E-domain (Mo insertase, GEPH-E). We showed that both reactions, which were initially studied using the individual domains, proceed approximately 100-fold more efficiently in the full-length enzyme utilizing physiological molybdate concentrations [15]. In addition, vertebrate gephyrin has an essential function in the central nervous system where it clusters inhibitory glycine and γ-amino butyric acid (GABA) type A receptors at postsynaptic synapses [16•].

Molybdenum enzymes

Today there are four different molybdenum enzymes known in humans: sulfite oxidase (SO), xanthine oxidoreductase (XOR), aldehyde oxidase (AO) and the mitochondrial amidoxime-reducing component, of which two isoforms (mARC1 and mARC2) are expressed (Figure 2a). While mARC proteins have been identified only a few years back [17], SO and XOR are well known for decades, they catalyze catabolic reactions in cysteine and purine metabolism, and their structure and reaction mechanism have been studied intensively [2]. In contrast, the physiological functions of AO and mARC enzymes remain to be identified, while both enzymes have been found to function in drug metabolism [17,18]. Based on the active site structure (Figure 2b), two families of eukaryotic Mo enzymes have been defined, the SO-type with a conserved cysteine forming the third equatorial sulfur-ligand of Moco and the xanthine oxidase (XO)-type, with the third equatorial sulfur as terminal sulfido ligand [19]. While AO belongs to the XO family, it remains unclear if mARC belongs to the SO family (as suggested by a functionally important and conserved cysteine residue) or represents a new family of Mo enzymes [20].

SO catalyzes the two-electron oxidation of sulfite (Figure 2a), derived from the oxidative catabolism of cysteine, to sulfate [19], representing the terminal step in the degradation of cysteine. SO is a homodimer with each monomer harboring an N-terminal cytochrome b_5-type heme domain, a catalytic Moco domain and a C-terminal dimerization domain. The enzyme is localized in the mitochondrial intermembrane space where electrons derived from sulfite oxidation are directly passed to the physiological electron acceptor cytochrome *c*. The maturation of mammalian SO has been clarified. It combines a conventional leader sequence-based translocation mechanism with the folding trap mechanism for which the presence of Moco is strictly required [21].

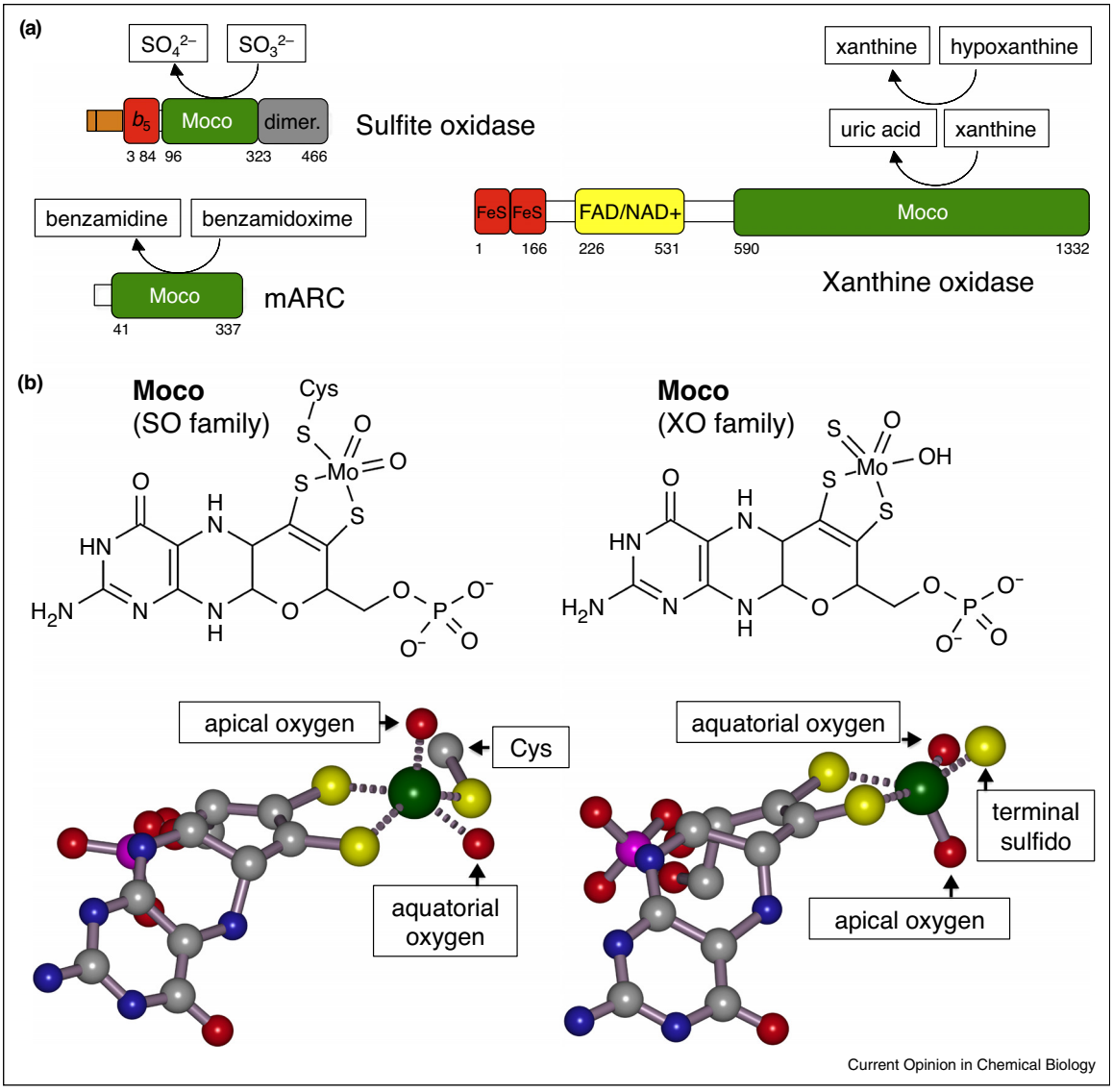
XOR catalyzes the terminal two steps in purine catabolism converting hypoxanthine to xanthine and xanthine to uric acid (Figure 2a). The enzyme forms a homodimer with a N-terminal domain binding two [2Fe-2S] clusters, a central FAD domain and a C-terminal Moco-binding domain that mediates dimerization. XOR is synthesized as dehydrogenase and transfers electrons from the substrate to NAD^+, however, posttranslational modifications convert the enzyme reversibly (oxidation of cysteines) or irreversibly (proteolytic cleavage) into an oxidase (XO). Recent crystal structures of XO in complex with substrate analogues have further advanced the understanding of the reaction mechanism [22]. In contrast to xanthine dehydrogenase, XO produces superoxide anions and hydrogen peroxide suggesting a role of XO in cell stress response and an important risk factor ischemia-reperfusion injury [23].

AO is structurally very similar to XOR and displays an active site, which can be superimposed to that of XO [24]. AO catalyzes the oxidation of aldehydes into carboxylic acids producing superoxide and hydrogen peroxide similar to XO [2•]. In contrast to XO, substrate specificity is much broader and includes heterocycles, purines, pteridines, which is why AO plays an important role in drug metabolism [18]. However, physiological substrates remain to be identified.

The most recently identified Mo enzymes are mARC1 and mARC2 [17], which were found to metabolize several N-hydroxylated compounds commonly used as pro-drugs [25] (Figure 2a). mARC enzymes are monomeric, are inserted into the outer mitochondrial membrane facing the cytosol where they interact with cytochrome b_5 and NADH/cytochrome b_5 reductase, building an electron transfer chain towards the Mo center [17,26]. The mARC-dependent metabolism of N-hydroxy-L-arginine, a precursor of classical nitric oxide (NO) synthase-dependent NO synthesis may suggest a role in the regulation of NO biosynthesis [17]. Recently, a function of mARC2 in lipogenesis has been reported [27].

Besides their primary substrates, Mo enzymes have been associated with moonlighting functions catalyzing

Figure 2



Mo enzymes and Moco structures. **(a)** Domain structure of chicken SO, bovine XO and human mARC1 as representative members of each type of human Mo enzyme. Domains are depicted as colored boxes. Moco: Moco-binding domain, dimerization domain (dimer.), b_5, heme cytochrome b_5 domain, FAD/NAD+ binding flavin domain. The different substrates are depicted. **(b)** Chemical and three-dimensional structures of Moco in the SO and XO family of Mo enzymes.

alternative reactions, such as the reduction of nitrite to NO, a process which is well known to regulate blood pressure, hypoxic vasodilation, cellular cyto-protection, and mitochondrial respiration and functions under hypoxic or exercise stress [28]. Given the broad substrate specificity of XO and AO, nitrite reduction has been first reported for those enzymes (summarized in [29]) but recently, specific activities and *in vivo* evidence for NO synthesis by SO and mARC enzymes have been reported too [30,31]. Both enzymes were able to reduce nitrite to NO *in vitro* and a fully reduced Mo(IV) active site was found to be required. Studies with fibroblasts lacking either SO or all Moco enzyme activities showed that nitrite-dependent cGMP synthesis (which requires NO) is mainly dependent on SO [31].

Moco and Mo enzyme deficiencies

Moco deficiency (MoCD) is a rare inborn error of metabolism causing the loss of all Mo enzyme activities and has been first reported in 1978. Patients present in their neonatal period feeding difficulties, therapy-resistant seizures, high pitch crying, followed by severe neurological abnormalities, lens dislocation of the eyes and major dysmorphic features of the head. Until today more than 100 cases with MoCD have been reported [32••], with most of them sharing a predominant deterioration of the

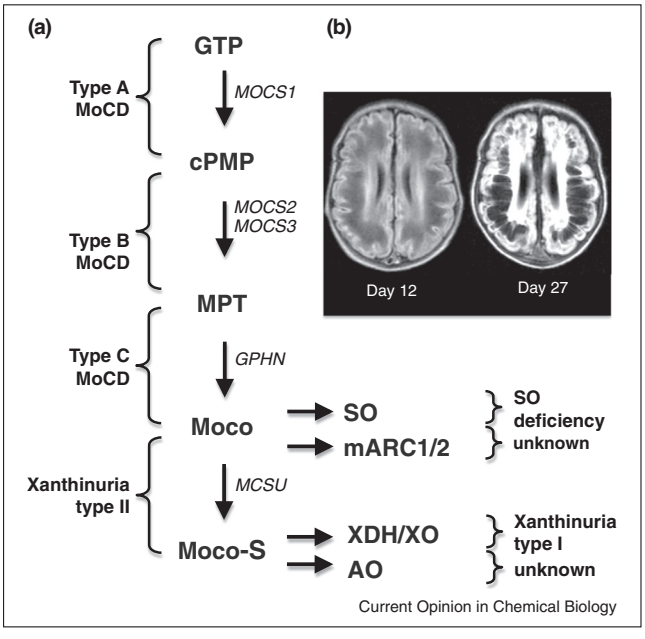
central nervous system. First symptoms are observed within days of life, starting with feeding difficulties followed by intractable seizures with an exaggerated startle reaction [3]. Disease progression is accompanied by psychomotor retardation due to progressive cerebral atrophy and ventricular dilatation. MRI features of the disease include global cerebral edema, cystic encephalomalacia, cortical and white matter atrophy, focal or bilateral changes within the globi pallidi and subthalamic regions, dysgenesis of the corpus callosum and ventriculomegaly [33•] (Figure 3). Patients that survive the neonatal period show essentially no neuronal development, are unable to make any coordinated movements, require tube feeding and show no signs of communication with their environment and usually die within their first years of life [32••], only very few cases are reported with mild symptoms or delayed disease onset [34].

Interestingly, most of the symptoms of MoCD are mirrored in isolated SO deficiency (Figure 3), which is caused by mutations in the *SUOX* gene [35] leading to sulfite accumulation. Therefore, SO is seen as most important Moco-dependent enzyme and sulfite accumulation presents the primary cause of neurodegeneration in both disorders. Sulfite accumulation is accompanied by changes in other S-containing metabolites such as cysteine, S-sulfocysteine, thiosulfate, homocysteine and taurine [3,36,37].

XOR deficiency results in the accumulation of xanthine in urine leading to a disease termed xanthinuria, which exist in two forms: xanthinuria type 1 and type 2 (Figure 3) [38,39]. Xanthinuria type 1 is caused by the loss of activity of XOR resulting in an accumulation of xanthine. In contrast, xanthinuria type 2 is caused by the simultaneous loss of activities of XOR and AO, which is caused by mutations in the *MCSU* gene encoding for a protein necessary for the sulfuration of Moco in enzymes of the XO family (Figure 3). In both types of xanthinuria, a very low level of plasma uric acid and high levels of xanthine are hallmarks of the disease. Patients of both groups have similar clinical presentation, mostly due to increased xanthine deposition [38]. However, the mechanism involved in the disease is less clear as some patients may develop symptoms, which may lead to acute renal failure while others remain asymptomatic.

MoCD is caused by mutations in any steps of the biosynthetic pathway of Moco. Already before the identification of Moco synthesis genes, biochemical studies identified two types of patients that were classified as MoCD 'type A' and 'type B' (Figure 3a). Co-culture studies with patient-derived fibroblasts revealed that 'type B' cells excreted the Moco precursor Z (later identified as cPMP), which was taken up by 'type A' cells. Following the identification of the underlying gene defects, it was found that MoCD 'type A' patients carry mutations in the *MOCS1* gene, while 'type B' patients are defective in *MOCS2* [5]. Surprisingly, no mutations were found in *MOCS3*. Mutations in the *GPHN* gene cause very severe forms of MoCD [5] due to impaired synaptic inhibition, a function for which gephyrin is also indispensible [16•]. Recently, various hemizygous defects in the *GPHN* gene were found to be causative in neuropsychiatric disorders such as epilepsy, schizophrenia and the development of seizures [40,41,42].

Figure 3



Types of MoCD and Mo-enzyme deficiencies. **(a)** Three major steps of Moco synthesis and the involved genes. MCSU encodes for the sulfurase, which catalyzes the sulfuration of Moco, which is essential for xanthine dehydrogenase (XDH)/XO and aldehyde oxidase (AO) activities. Patients with mARC and AO deficiencies have not been reported yet. **(b)** MRI scans of a MoCD type A patient recorded at an age of 12 and 27 days showing the rapidly progressing brain damage resulting in brain atrophy and cystic changes in the cerebral cortex. *Source*: Figure has been modified from [3].

Disease mechanism in MoCD and SO deficiency

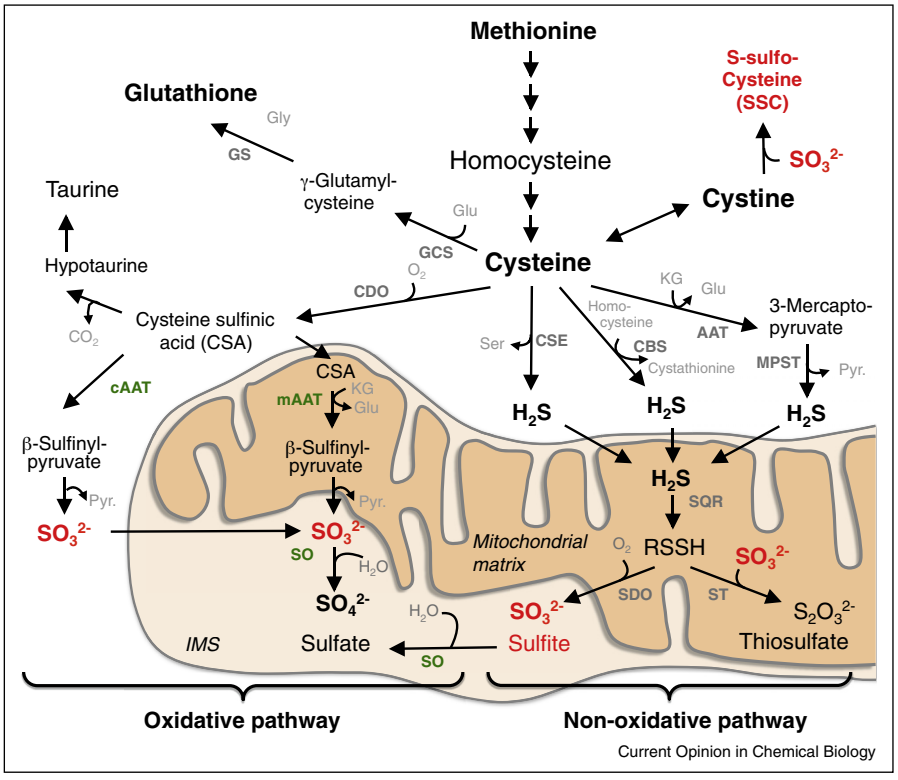
In the absence of SO activity (as seen in MoCD and SO deficiency), sulfite accumulates within the cell and has been found to increase reactive oxygen species [43]. Sulfite inhibits glutamate dehydrogenase, which in turn decreases ATP synthesis in mitochondria when respiring on glutamate. Accumulated sulfite passes the plasma-membrane and spreads throughout the body via the circulation. Within the extracellular space, it can reduce disulfide bridges, primarily in membrane proteins, thus affecting protein folding, stability, and activity. The first sulfite scavenging mechanism is the reaction with cystine leading to the formation of S-sulfocysteine (SSC), which is highly abundant in MoCD and SO deficiency patients

(Figure 4). SSC is structurally similar to glutamate and able to bind to N-methyl-D-aspartate (NMDA) receptors and proposed to be the cause of seizures and subsequent brain damage in the patients. In fact, early studies in rats demonstrated that SSC induces the same type of brain damage as glutamate, suggesting an excitotoxicity-based disease mechanism. In this context we have studied SSC-mediated neuronal cell death and found a receptor-mediated calcium influx followed by calpain activation and proteolytic cleavage of synaptic proteins (Kumar et al. unpublished results). Sulfite-dependent SSC formation results in cystine depletion, which directly impacts glutathione synthesis [44] (Figure 4), the major antioxidant in neuronal tissue and most abundant low-molecular-weight thiol in animal cells. Furthermore, the observed accumulation of thiosulfate suggests an increased reaction of sulfite with protein-bound persulfides, the latter being dependent on H_2S, which is derived from the non-oxidative cysteine elimination pathway [45]. The important function of H_2S as a signaling molecule in the brain has only recently been recognized [46] and factors leading to reduced H_2S levels are believed to contribute to the pathogenesis of age-associated neurodegeneration such as Huntington's disease [47••].

Treatment options

In light of the well-recognized cause of neurodegeneration in SO deficiency and MoCD, early attempts to cure the disease aimed to reduce the level of sulfite by dietary restriction. While these attempts showed only moderate clinical success, biochemical responses were observed, showing a reduction in sulfite excretion. In a recent case with a delayed onset of SO deficiency, dietary restriction was very effective in stabilizing the patient and to suppress seizure activity [48]. Based on the underlying mutation one

Figure 4



Cysteine catabolism in humans. Cysteine can be absorbed from the diet or synthesized from methionine through several intermediates of the transmethylation and transsulfuration pathway. Cysteine is the precursor of glutathione (GSH), which is synthesized by two enzymatic reactions catalyzed successively by γ-glutamylcysteine synthetase (GCS) and glutathione synthetase (GS). The catabolism of cysteine follows two major routes: an oxidative pathway leading to the formation of sulfate and taurine as sulfur-containing endproducts. While the SO reaction takes place in the mitochondrial intermembrane space (IMS), the preceding synthesis of β-sulfinyl pyruvate can either take place in the cytosol (catalyzed by cytosolic AAT) or in the mitochondrial matrix (catalyzed by mitochondrial mAAT). Depending on the site of synthesis, cysteine sulfinic acid might need to translocate to the matrix and sulphite might need to pass the inner mitochondrial membrane. Alternatively, a non-oxidative pathway leads to hydrogen sulfide formation, which is entering the mitochondrial matrix and can be further converted to sulphite and thiosulfate. The enzyme abbreviations used are: CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase (cystathionase); CDO, cysteine dioxygenase; CSD, cysteinesulfinate decarboxylase; AAT, aspartate aminotransferase; SO, sulfite oxidase; MPST, 3-mercaptopyruvate sulfurtransferase; SQR, quinone oxidoreductase; SDO, sulfur dioxygenase; ST, sulfur transferase.

can argue that dietary restricting can suppress disease phenotypes in mild forms of SO deficiency.

Symptomatic treatment of patients was in most cases restricted to seizure control using phenobarbital and midazolam, both of which targeting GABAergic synaptic transmission. One patient case was treated with an NMDA receptors antagonist (dextromethorphan) showing moderate improvement of seizures in the patient that had very progressed MoCD [49]. In light of recent findings that show an excitotoxicity-based mechanism of neuronal cell death (Kumar et al. unpublished results) [44], early intervention with drugs blocking NMDA receptor activation may slow down neurodegeneration in MoCD.

The fact that most MoCD patients carry mutations in *MOCS1* [50] motivated us to develop a *mocs1* knockout mouse model reproducing the human disease [51]. As these animals were unable to synthesize cPMP, we developed a fermentation and purification procedure in *E. coli* [52] to obtain purified cPMP for treatment studies. cPMP-treated *Mocs1*-deficient mice developed normally, reached adulthood, were fertile and not distinguishable from their wild-type littermates [53]. Biochemically, a dose and treatment interval-dependent restoration of Moco synthesis as well as Mo-enzyme activity was observed.

Based on the promising results of cPMP substitution therapy in *mocs1*-deficient mice, treatment of a first *MOCS1* patient was initiated on day 36 of life [54]. The starting dose of 80 μg cPMP per kg body weight was extrapolated from the animal studies and was sufficient to induce a remarkable and sustained improvement of MoCD biomarkers within days after treatment. Following dose adjustments to 240 μg/kg body weight, normalization of biomarkers was observed. Clinical improvement was recognized 48 hours after treatment start; convulsions and twitching disappeared within the first two weeks and epileptic discharges were reduced [54]. Based on that case, a treatment plan for future patients was developed and recently we reported a prospective cohort study in 16 neonates diagnosed with MoCD [55]. Out of 11 type A patients, treatment in eight patients was continued for up to five years, all showing normalization of biomarkers. Clinically, convulsions were either completely suppressed or substantially reduced. Three patients treated early, remained seizure-free and showed near-normal long-term development. In conclusion, cPMP substitution is the first effective therapy for patients with MoCD type A and, when treated early enough, it can greatly improve neurodevelopmental outcome. Therefore, the possibility of MoCD type A needs to be urgently explored in every encephalopathic neonate to avoid any delay in appropriate cPMP substitution, and to maximize treatment benefit.

Patients suffering from SO deficiency and MoCD type A do not benefit from cPMP therapy and therefore require alternative treatment options. In a recent study we explored the concept of enzyme substitution therapy by first establishing a simplified SO enzyme (by deleting the heme domain) that renders SO to accept molecular oxygen as electron acceptor [56••]. Such a truncated from of SO does not require the mitochondrial translocation and therefore allows intravenous infusion as enzyme substitution therapy. To increase the half-life of SO, we applied surface PEGylation, which caused an increased conformational stability with similar kinetic properties as compared to wild type SO [56••]. Future animal studies are required to explore the possibility of SO-based enzyme substitution therapies of sulfite toxicity disorders.

Outlook

The biosynthesis of Moco is well understood. While a deficiency in one of the four Moco-dependent enzymes can either be asymptomatic in some cases (XOR deficiency) or lethal in other cases (SO deficiency), MoCD, however, is in nearly all cases a severe inborn error in metabolism and characterized by a rapidly progressing neurodegeneration. In recent years, knowledge regarding the underlying mechanism causing severe brain damage has accumulated. However, future studies are needed to identify key players in metabolism that initiate neuronal cell death. In light of the important role of SO, mitochondrial dysfunction could represent a crucial entry point for signals contributing to cell death. This knowledge will be important for the treatment of future patients suffering of MoCD by providing new concepts in protecting the brain from rapidly progressing brain injury. Besides cPMP, alternative therapies need to be developed for the treatment of MoCD 'type B' and SO deficiency. The physiological roles of AO and mARC are still poorly understood and novel animal models are instrumental to identify their primary function in metabolism. Finally, the physiological relevance of nitrite reduction by Mo enzymes needs to be further explored and might provide an attractive target for the development of future drugs in the treatment of cardiovascular disorders.

Acknowledgements

I am very grateful to all my past and current coworkers and collaborators for their hard and enthusiastic work. I am particularly thankful to my longstanding collaborator Dr. Jose Angel Santamaria Araujo for his work on cPMP, its production and continuous supply to patients. I would like to thank my previous postdocs, Dr. Abdel Ali Belaidi and Dr. Borislav Dejanovic for their fundamental contributions to the fields of Moco biosynthesis and therapy as well as gephyrin-mediated synaptogenesis, respectively. I thank Dr. Katrin Schrader for her continuous support through many years in Cologne that enabled to run the lab smoothly. The work related to this review has been funded by various grants of the German Science Foundation (DFG), the EU FP7, the Federal Ministry for Education and Research (BMBF), the Center for Molecular Medicine Cologne (CMMC), and the Fonds der Chemischen Industrie (FCI).

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Papers of particular interest, published within the period of review, have been highlighted as:

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- •• of outstanding interest

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Following the primary review on mononuclear molybdenum enzymes in 1996 (Ref. [19]), this updated review provides an excellent overview on the reaction mechanism, structure and function of all families of molybdenum enzymes. Special emphasis is given to xanthine oxidoreductase and sulphite oxidase. In my view, there is no other review available, which is of any comparable death and comprehensiveness. In particular many figures provide an outstanding level of comparison and systematic review of individual studies performed on molybdenum enzymes. The previous Chemical Review on Mo enzymes represents the most-cited work in the field.

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Following the identification of 3′,8-cyclo-7,8-dihydro-guanosine 5′-triphosphate (3′,8-cH2GTP) as reaction intermediated in cPMP synthesis, two laboratories (Yokoyama and Begley labs) collected experimental evidence, which part of the multistep cPMP biosynthesis is catalyzed by which of the two involved proteins (MoaA and MoaC). This publication provides strong and comprehensive evidence that (3′,8-cH2GTP) is the reaction product of MoaA function by elucidating the crystal structure of MoaC in complex with its substrate 3′,8-cH2GTP. Furthermore, turn over of 3′,8-cH2GTP by MoaC was characterized.

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