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Review

Molybdenum enzymes, their maturation and molybdenum cofactor biosynthesis in *Escherichia coli* ☆

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ABSTRACT

Molybdenum cofactor (Moco) biosynthesis is an ancient, ubiquitous, and highly conserved pathway leading to the biochemical activation of molybdenum. Moco is the essential component of a group of redox enzymes, which are diverse in terms of their phylogenetic distribution and their architectures, both at the overall level and in their catalytic geometry. A wide variety of transformations are catalyzed by these enzymes at carbon, sulfur and nitrogen atoms, which include the transfer of an oxo group or two electrons to or from the substrate. More than 50 molybdoenzymes were identified in bacteria to date. In molybdoenzymes Mo is coordinated to a dithiolene group on the 6-alkyl side chain of a pterin called molybdopterin (MPT). The biosynthesis of Moco can be divided into four general steps in bacteria: 1) formation of the cyclic pyranopterin monophosphate, 2) formation of MPT, 3) insertion of molybdenum into molybdopterin to form Moco, and 4) additional modification of Moco with the attachment of GMP or CMP to the phosphate group of MPT, forming the dinucleotide variant of Moco. This review will focus on molybdoenzymes, the biosynthesis of Moco, and its incorporation into specific target proteins focusing on *Escherichia coli*. This article is part of a Special Issue entitled: Metals in Bioenergetics and Biomimetics Systems.

1. Molybdenum in biological systems

Molybdenum was first discovered to be present in molybdenite (MoS_2) by Scheele in 1778–79 and later isolated from molybdenite by Hjelm in 1782, who named it molybdenum (from greek molybdos, meaning lead or lead-like). The first biological function was proven by Bortels in 1930 [1], who showed that it acts as a catalyst in the fixation of nitrogen by *Azotobacter chroococum*, and 23 years later, in 1953 it was reported that molybdenum also has a direct role in the metabolism of animals and humans [2–4]. The biological active form of molybdenum is the soluble oxyanion molybdate. Although only a minor constituent of the earth's crust, molybdenum is widely bioavailable because of the high solubility of molybdate salts in water; molybdenum is, for example, the most abundant transition metal in seawater [5].

Molybdenum is the only 4d transition metal required for biological systems which forms part of the active site of molybdoenzymes that excecute key transformations in the metabolism of nitrogen, sulfur and carbon compounds [6]. Molybdenum as the catalytic center in enzymes has a chemical versatility that is useful to biological systems: it is redox-active under physiological conditions (ranging between the oxidation states VI and IV); since the V oxidation state is also accessible, the metal can act as transducer between obligatory two-electron and one-electron oxidation-reduction systems and it can exist over a wide range of redox potentials [7]. The catalyzed reactions are in most cases oxo-transfer reactions, e.g. the hydroxylation of carbon centers and the physiological role is fundamental since the reactions include the catalysis of key steps in carbon, nitrogen and sulfur metabolism. In the context of availability, its concentration is 10^{-7} mol/l in seawater, which is equal to other transition metals including iron [2,5]. Once molybdate enters the cell it is subsequently incorporated by complex biosynthetic machineries into metal cofactors. These metal cofactors are then incorporated into different enzymes and these molybdenum enzymes are found in nearly all organisms, with *Saccharomyces cerevisiae* as a prominent eukaryotic exception [8]. There are two distinct types of molybdoenzymes: Molybdenum nitrogenase has a unique molybdenum–iron–sulfur cluster, the $[Fe_4S_3]$-(bridging-S)$_3$-$[MoFe_3S_3]$ center called FeMoco [9]. Nitrogenase catalyzes the reduction of atmospheric dinitrogen to ammonia. All other molybdoenzymes are oxidoreductases that transfer an oxo group or two electrons to or from the substrate [6]. They have a molybdenum cofactor (Moco, Fig. 1) in which molybdenum is coordinated to a dithiolene group on the 6-alkyl side chain of a pterin called molybdopterin (MPT) [10]. In contrast to the multinuclear FeMoco in nitrogenase, the active site of mostly all other well characterized Moco containing enzymes is generally mononuclear, with a single equivalent of the metal (carbon monoxide dehydrogenase from

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Oligotropha carboxydovorans is so far the only exception [11]). In the redox reactions catalyzed generally by molybdoenzymes electron transfer is linked with proton transfer. Other metal complexes usually do not display the appropriate redox potentials in combination with the appropriate acid-base properties. Most of the other metal centers in biology, including those of iron and copper, participate only in electron transfer [2]. Moreover, few metals other than molybdenum can effect oxygen transfer. The ability of molybdenum to exist in various oxidation states under physiological conditions and to couple oxide or proton transfer with electron transfer makes it the metal of choice for the reactions in which it participates. Here, we will focus on the molybdoenzymes identified and characterized in *Escherichia coli*, since this is the best characterized system and an overview concentrating on this organism has not been reported so far. Additionally, numerous other reviews exist which cover a broader view of Moco biosynthesis and molybdoenzymes in bacteria and eukaryotes.

2. Molybdate uptake and regulation

Molybdenum is transported in the form of the oxyanion molybdate (MoO_4^{2-}). In *E. coli* molybdate may be taken up through three transport systems [12,13]: the high-affinity ModABC system [14], the CysPTWA sulfate-thiosulfate permease with low affinity [15], and a non-specific low-efficiency anion transport system that requires high molybdate concentrations, and which also transports sulfate, selenate, and selenite [15].

The high-affinity molybdate transport system ModABC belongs to the ATP-binding cassette (ABC) superfamily of transporters found in prokaryotes and eukaryotes [16–18]. In *E. coli*, this ABC transporter consists of two integral membrane proteins ModB and two hydrophilic peripheral membrane proteins ModC that are ATP-binding proteins. In addition, a periplasmic binding protein ModA captures the substrate (K_D of 20 nM, [19]) and delivers it to the transporter complex in the inner membrane. Both molybdate and tungstate bind to the *E. coli* ModA protein in the periplasm as a tetrahedral complex which is held by seven hydrogen bonds formed between the oxygen of the bound anion and the protein groups from two domains [20]. ModB is the transmembrane protein for transporting molybdate through the membrane. The crystal structure of the homologous tungsten transporter WptABC of *Archeoglobus fulgidus* showed that the anion is transported through a large cavity within WptB, building the translocation pathway [18,21]. The MobB–ModC interface transmits critical conformational changes, thus coupling ATP binding and hydrolysis to transport. For molybdate-dependent regulation, the *modABC* operon expression is negatively controlled by the ModE protein, which binds to the operator region of the *modABC* operon in its molybdate-bound form [22]. ModE also enhances the transcription of molybdenum-dependent enzymes like dimethylsulfoxide reductase (DMSO) reductase [23], nitrate reductase [24], formate hydrogenlyase [24] and also of the molybdenum cofactor biosynthesis operon *moaABCDE* [25]. Additionally, the operons for a peptide transporter, *oppABCDF*, and for proteins involved in nucleoside catabolism, *deoCABD*, are also regulated by ModE [26]. ModE is a homodimer and each monomer can be subdivided into four structural domains: the N-terminal DNA-binding domain, a linker domain and two molybdate-binding (Mop) domains [27]. ModE binds two molybdate molecules per dimer (K_D of 0.8 μM) and molybdate binding subsequently results in extensive conformational changes of the domain thus enabling DNA binding [27].

A Moco-sensing riboswitch candidate has been identified in *E. coli* and other bacteria [28]. In *E. coli*, the highly conserved RNA motif is located upstream of genes for Moco biosynthesis *moaABCDE* (see below), thus providing an additional level of regulation in response to the actual cofactor coordinating the molybdenum atom [28].

3. The structure of the molybdenum cofactor

In the large group of molybdoenzymes coordinating Moco, the molybdenum atom is coordinated to the dithiolene group on the

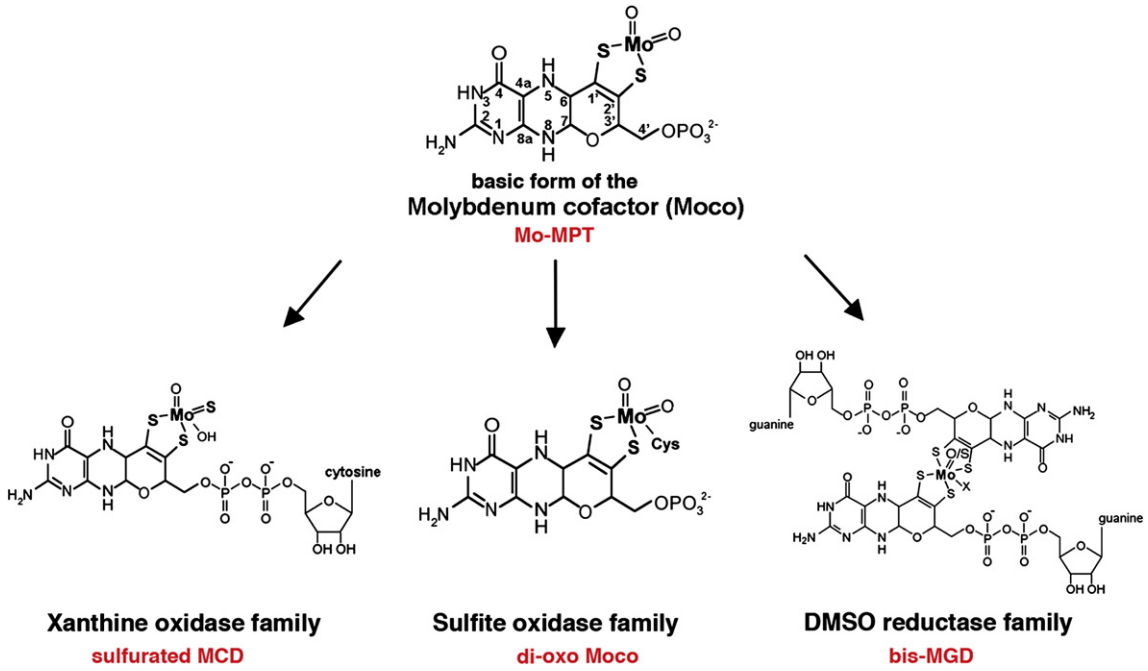

Fig. 1. Different structures of the molybdenum cofactor in *E. coli*. The basic form of the molybdenum cofactor is a 5,6,7,8-tetrahydropyranopterin (MPT) with a unique dithiolene group coordinating the molybdenum atom. This structure is referred to Mo-MPT in this review. Moco exists in different variants and is divided into three molybdenum-containing enzyme families according to the coordination at the molybdenum atom: the xanthine oxidase, sulfite oxidase, and DMSO reductase families. In *E. coli*, the xanthine oxidase family contains the sulfurated molybdopterin cytosine dinucleotide cofactor (MCD). The sulfite oxidase family is characterized by a di-oxo Moco with an additional protein ligand, which usually is a cysteine. The DMSO reductase family contains two MGDs ligated to one molybdenum atom with additional ligands being an O/S, and a sixth ligand X, which can be a serine, a cysteine, a selenocysteine, an aspartate or a hydroxide and/or water molecule.

6-alkyl side chain of the tricyclic pyranopterin, molybdopterin (MPT) also referred to as metal-binding pterin [9]. The chemical nature of Moco has been determined by Rajagopalan and Johnson in 1992 [10]. They postulated a structure of the cofactor consisting of a pterin derivative, with the pterin ring substituted at position 6 with a phosphorylated dihydroxybutyl sidechain containing a cis-dithiolene bond. The sulfur atoms of the dithiolene group were proposed to coordinate the molybdenum atom, with a stoichiometry of one MPT per Mo. In bacteria like *E. coli*, additional variability of the Moco is achieved by attachment of a second nucleotide, GMP or CMP to the phosphate group of MPT (Fig. 1) [29]. The first crystal structure of a molybdoenzyme, the aldehyde ferredoxin oxidoreductase (AOR) from *Pyrococcus furiosus* [30], established the general validity of this model, with the additional feature that the dihydroxybutyl sidechain actually forms a pyran ring by attack of the 3′-hydroxyl group on C7 of the pterin. Subsequently this tricyclic form of the pterin was also observed in the crystal structures of the molybdoenzymes solved thereafter [31]. As demonstrated by the crystal structures of several molybdoenzymes (AOR from *P. furiosus* [30], aldehyde oxidoreductase from *Desulfovibrio gigas* [32], DMSO reductase from *Rhodobacter sphaeroides* [33]), the tricyclic MPT system is distinctly nonpolar. In particular, the pyran ring adopts a half-chair conformation in these structures that deviates significantly from the plane of the pterin system. Additionally, the structures suggested that the MPT is in the fully reduced tetrahydropterin oxidation state [31]. In molybdoenzymes like DMSOR or AOR, the metal atom is coordinated by two MPTs; thus, these cofactors are referred to as bis-Moco. In the original structure determinations of the molybdopterin guanine dinucleotide (MGD) containing DMSO reductase from *Rhodobacter sphaeroides* (DorA), the two pterins were arbitrarily referred to as "P" and "Q," depending on their location to the following redox-centers (e.g. FeS clusters), as proximal and distal pterin [33].

In total, the molybdenum cofactor exists in three basic configurations at the molybdenum atom in *E. coli*, which has been determined by a combination of rigourous biochemical studies and confirmed by the emergence of a large amount of structural data [31]. Thus, the molybdoenzymes binding the different forms of Moco were historically categorized into three families based on the type of cofactor bound, which is mainly classified by the ligands at the molybdenum atom (Fig. 1): the xanthine oxidase family, the sulfite oxidase family and the DMSO reductase family [6]. Newer classifications also include the tungsten-containing enzymes in one enzyme family [7,34]. Since *E. coli* does not contain tungsten-containing enzymes, we will stick to the original classification with three enzyme families in this review. Further, we will describe the members of each family with examples of the molybdoenzymes identified in *E. coli* (Table 1).

4. The three families of molybdoenzymes in *E. coli*

4.1. The xanthine oxidase family

The xanthine oxidase (XO) family is characterized by an MPT-$Mo^{VI}OS(OH)$ core in the oxidized state, with one MPT equivalent coordinated to the metal (Fig. 1) [6]. The additional sulfido-group is cyanide labile [35]. Removal of the sulfido group results in formation of an inactive desulfo-enzyme, with an oxygen ligand replacing the sulfur at the Mo active site [36]. The enzymes of this family are involved in two-electron transfer hydroxylation and oxo-transfer reactions with water as the source of oxygen. Among the members of the xanthine oxidase family in *E. coli* are the xanthine dehydrogenase XdhABC, the periplasmic aldehyde oxidoreductase PaoABC, and the so far uncharacterized xanthine dehydrogenase homologue XdhD (Table 1) [37]. The *paoABCD* operon encodes for a molybdenum-containing iron–sulfur flavoprotein which is located in the periplasm (Fig. 2) [38]. The 135 kDa enzyme comprises a noncovalent ($\alpha\beta\gamma$) heterotrimer with a large (78.1 kDa) molybdenum cofactor (Moco)-containing PaoC subunit, a medium (33.9 kDa) FAD-containing PaoB subunit, and a small (21.0 kDa) 2x[2Fe2S]-containing PaoA subunit, which also contains the Tat-leader peptide for the localization to the periplasm. PaoD is not a subunit of the mature enzyme, and the protein is expected to be involved in Moco modification and insertion into PaoABC. Analysis of the form of Moco present in PaoABC revealed the presence of the molybdopterin cytosine dinucleotide cofactor (MCD) [38]. Kinetic characterization of the enzyme showed that PaoABC converts a broad spectrum of aldehydes, with a preference for aromatic aldehydes. Ferredoxin instead of NAD^+ or molecular oxygen was used as terminal electron acceptor. Complete growth inhibition of *E. coli* cells devoid of genes from the *paoABC* operon was observed by the addition of cinnamaldehyde to a low-pH medium. This finding showed that PaoABC might have a role in the detoxification of aromatic aldehydes for *E. coli* under certain growth conditions [38].

Studies on the XdhABC protein have not been published so far. However, the purified protein can be obtained from Sigma. Characterization of the protein showed that the cytoplasmic protein is an $(\alpha\beta\gamma)_2$ heterohexamer that contains 2x [2Fe2S] clusters bound to the XdhC subunit, FAD bound to the XdhB subunit and the MCD cofactor bound to the XdhA subunit (Table 1, Fig. 2) [37,39]. Analysis of the substrate specificity showed that it predominantly converts xanthine and hypoxanthine to uric acid (unpublished results). In contrast, XdhD is an unusual XO-family enzyme which has not been characterized so far. All attempts to purify the enzyme after homologous expression in *E. coli* failed (unpublished results). XdhD shares homologies to xanthine dehydrogenases; however, it encodes for a fusion protein composed of the FeS cluster-containing subunit and the Moco-containing subunit. Additionally, the conserved cysteines for coordinating the [2Fe2S] clusters are missing [37,39]; thus, the enzyme should be devoid of FeS clusters. Upstream of *xdhD* a coding region for an FAD-binding protein (YgfM) was identified. It was shown that YgfM and XdhD (YgfN) form a complex and were found to be significant iron containing proteins under elevated iron conditions (Table 1, Fig. 2) [40]. However, so far XdhD could not be expressed in an active form (unpublished results); thus, the role of this enzyme for *E. coli* remains unknown.

4.2. The sulfite oxidase family

Enzymes of the sulfite oxidase (SO) family coordinate a single equivalent of the pterin cofactor with an MPT-$Mo^{VI}O_2$ core in its oxidized state (Fig. 1), and usually an additional cysteine ligand which is provided by the polypeptide [6]. Members of this family catalyze the transfer of an oxygen atom either to or from the substrate. Among the members of this family in *E. coli* is solely the YedY protein, the catalytic subunit of a sulfite oxidase homologue [41]. So far, all members of this family including YedY contain the MPT-form of Moco without an additional dinucleotide. The *yedY* gene was identified in a screening for genes of molybdoenzymes [42]. Compared to molybdoproteins with a known function, the amino acid sequence of YedY is most homologous to the Moco-binding domain II of SO. YedY has a twin-arginine translocation (Tat) signal peptide, which targets the folded protein for translocation across the cytoplasmic membrane. Together with *yedY*, the *yedZ* gene forms an operon. YedZ is a membrane spanning cytochrome b like protein, which probably provides both a membrane anchor and a redox partner for the soluble YedY (Table 1, Fig. 2). The YedYZ complex thus constitutes a heme-molybdoenzyme similar to the SO subunits I and II. Orthologues of the YedYZ complex are also present in other Gram-negative bacteria, including many pathogenic strains. Recently, the crystal structure of YedY was solved at 2.5 Å resolution [42]. The overall fold of the Moco binding site is similar in both YedY and SO, but the substrate binding site in YedY was shown to be different. The YedY substrate site is more similar to those of the bacterial reductases like DMSO and trimethylamine N-oxide (TMAO) reductases. Accordingly, YedY shows reductase activity only with

Table 1
Overview on the *E. coli* molybdoenzymes, their subunits, tageting to the periplasm and the involved chaperones required for maturation.

Mo-enzyme	Subunits	Substrates	Tat-subunit	Chaperone
DMSO reductase family				
Nitrate reductase A	NarGHI	Nitrate	–	NarJ
Nitrate reductase Z	NarZYV	Nitrate	–	NarW
Peripl. Nitrate reductase	NapABCGH	Nitrate	NapA	NapD
TMAO reductase A	TorAC	TMAO	TorA	TorD
TMAO reductase Z	TorZY	TMAO	TorZ	?
DMSO reductase	DmsABC	DMSO	DmsA	DmsD
Formate dehydrogenase N	FdnGHI	Formate	FdnG	FdhD
Formate dehydrogenase O	FdoGHI	Formate	FdoG	FdhD
Formate dehydrogenase H	FdhF	Formate	–	FdhD
Biotin sufoxide reductase	BisC	Biotin S oxide	–	–
Selenate reductase	YnfEFGH	Selenate	YnfE, YnfF	DmsD
?	YdhYVWXUT	?	YdhX	?
?	YdeP	?	–	?
Xanthine oxidase family				
Aldehyde oxidoreductase	PaoABC	Aldehydes	PaoA	PaoD
Xanthine dehydrogenase	XdhABC	Purines	–	YqeB
?	XdhD, YgfM	?	–	YqeB
Sulfite oxidase family				
Sulfite oxidase	YedYZ	?	YedY	–
?	YcbX	HAP	–	–
?	YiiM	HAP	–	–

–: periplasmic molybdoenzyme, no Tat leader present or no chaperone identified.
?: function unknown or not clear which chaperone is involved in maturation.
HAP: 6-N hydroxylaminopurine.

substrates such as TMAO and DMSO, and no oxidase activity with sulfite. Substrate screenings revealed catalytic activity generally only with S- and N-oxides. The turnover rate of YedY, however, is much lower than those of DMSO and TMAO reductases in *E. coli*. Hence, the function and substrate of YedYZ in *E. coli* still remain unknown. XAS analysis revealed an overall similar structure of the molybdenum site in YedY and hSO with respect to the two sulfur ligands from the MPT moiety of Moco and the S_{Cys} ligand [43]. Additionally, the molybdenum in YedY carried two oxo groups, as was previously found. However, in the crystal structure of YedY, the axial oxygen ligand of molybdenum clearly was assigned to an oxo group, but the nature of the equatorial oxo-ligand remained unclear [42]. EXAFS data showed that the ligand is either an $-O^-$ or an -OH ligand [43]. This leads to the conclusion that although YedY and hSO belong to the same family of molybdoenzymes, the differences in the structure of its molybdenum site may be related to the diverging activity and substrate specificity of YedY. The geometry of the active site and the conserved amino acids close to the molybdenum site may establish a hydrogen-bonded network, involving the equatorial hydroxyl ligand of molybdenum and a nearby water molecule, which, in turn, favors the Mo^V oxidation state and governs interaction of the Mo-OH/O^- species with the substrate. This substrate may be expected to be oxidized in single-electron abstraction steps, involving the reduction of Mo^V to Mo^{IV} and electron transfer from YedY to its counterpart, the YedZ protein in the membrane [43]. No other members of the sulfite oxidase family were identified in *E. coli* so far.

4.3. The DMSO reductase family

The DMSO-reductase family is diverse in both structure and function, but all members have two equivalents of the pterin cofactor bound to the metal [6]. The molybdenum coordination sphere is usually completed by a single Mo = O group with a sixth ligand in the MPT_2-$Mo^{VI}O(X)$ core (Fig. 1); however, a sulfido ligand has been recently described to replace the oxo-group in formate dehydrogenase-H from *E. coli* [44]. The sixth ligand, X, can be a serine, a cysteine, a selenocysteine, an aspartate or a hydroxide and/or water molecule. The reactions catalyzed by members of this family frequently involve oxygen-atom transfer, but dehydrogenation reactions also occur. Members of the DMSO reductase family are not present in eukaryotes and include, among other enzymes, the dissimilatory nitrate reductases, formate dehydrogenases, trimethylamine-N-oxide (TMAO) reductases, DMSO reductase and biotin sulfoxide reductases of *E. coli* (Table 1, Fig. 2). All these enzymes have two equivalents of MGD (bis-MGD) bound to the molybdenum at the active site (Fig. 1) [6]. In the absence of oxygen and in the presence of their respective substrates, these enzymes generally serve as terminal reductases in *E. coli*; however, they generally can catalyze sulfur- or proton transfer reactions, hydroxylations and non-redox reactions [34]. Another striking feature of most *E. coli* molybdoenzymes from this family is that they are either membrane-associated or located in the periplasm. Proteins are targeted to the Tat pathway by N-terminal signal peptides harbouring consecutive, essentially invariant, arginine residues within an SRRxFLK consensus motif [45]. This signal sequence has been identified in *E. coli* in the DMSO reductase and a homologue (DmsA, YnfE/F), formate dehydrogenases (FdnG and FdoG), periplasmic nitrate reductase and its electron donor- protein (NapA and NapG), TMAO reductase and a homologue (TorA and TorZ) [46]. Most molybdoenzymes in *E. coli* are part of respiratory systems, and sometimes more than one respiratory system is produced for a given substrate. Reduction of nitrate can be carried out by at least three respiratory systems [47]: at high concentrations of nitrate, only the membraneous NarGHI system is synthesized [48], whereas at very low concentrations the Nap system is produced [49]. The operon encoding a third system, *narZYWV*, is expressed during the early stationary phase independent of the presence of nitrate [50]. TMAO is reduced to the volatile compound trimethylamine (TMA) by at least two respiratory systems, the TorCAD and DmsABC systems [51,52]. Formate dehydrogenase catalyzes the oxidation of formate to CO_2 and H^+. *E. coli* contains two structurally related but differentially expressed respiratory formate dehydrogenases: formate dehydrogenase-O and formate dehydrogenase-N [53]. FdhGHI is a component of the nitrate respiratory pathway, where, under anaerobic conditions, formate oxidation is coupled to nitrate reduction (NarG) via lipid-soluble quinone [53,54]. FdoGHI and NarZYV are the corresponding isoenzymes that are additional present under aerobic conditions to ensure rapid adaptation during a shift from aerobiosis to anaerobiosis [55,56]. A third formate dehydrogenase (FdhF) is linked to the hydrogenase 3 and forms the formate-hydrogen lyase system [57]. A characteristic of formate dehydrogenases is an intrinsic selenocysteine residue that acts as ligand to molybdenum in the bis-MGD cofactor (Fig. 3). Additionally, *E. coli* contains the biotin sulfoxide reductase BisC, which is localized in the cytoplasm and converts biotin sulfoxide to biotin [58]. The selentate reductase comprising YnfEF protein is transcribed by the *ynfEFGHdmsD* operon. YnfE and YnfF share homologies to the DmsA subunit, YnfG to the DmsB subunit and YnfH to the DmsC subunit of DMSO reductase [59]. The protein has been described as a selenate reductase, since deletion mutants are unable to reduce selenate to selenium. The DmsD protein was shown to be involved in the maturation of DmsABC [60].

4.4. Uncharacterized molybdoenzymes in E. coli

So far uncharacterized enzymes with putative binding sites for Moco are YdhV and YdeP in *E. coli* (Table 1). YdeP is transcribed in a single transcription unit and encodes for a putative oxidoreductase binding bis-MGD. YdeP overproduction causes resistance to low pH [61]. It was predicted that the protein contains an additional [4Fe4S] cluster.

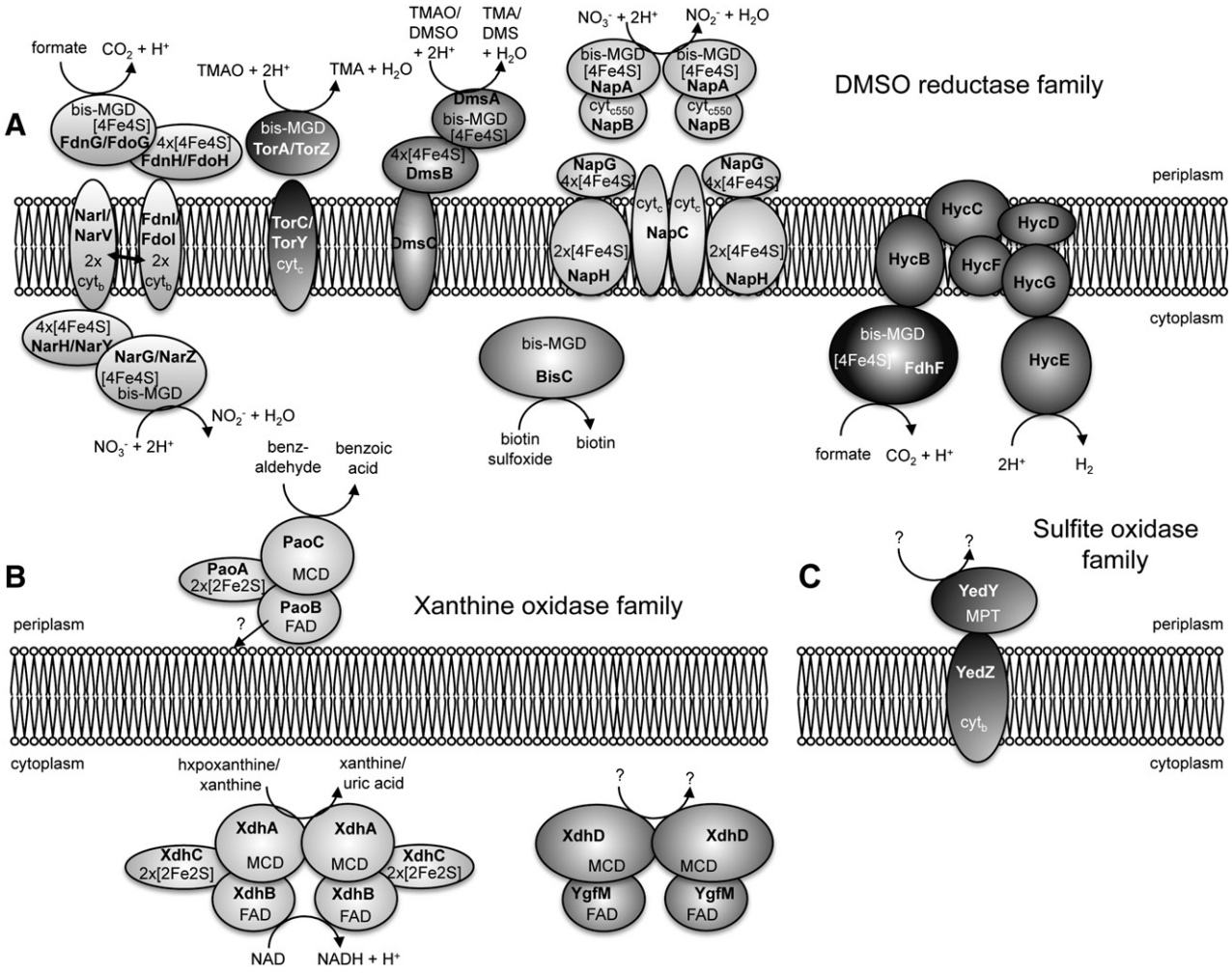

Fig. 2. Proposed localization of *E. coli* molybdoenzymes with known function. (A) Localization of enzymes of the DMSO reductase family. FdnGHI/FdoGHI are components of the nitrate respiratory pathway, in which formate oxidation is coupled to nitrate reduction (NarGHI/NarVYZ) which are coupled by a redox loop (shown by the arrow). TMAO is reduced to TMA by at least three respiratory systems, TorCA/TorYZ and DmsABC (also reducing DMSO to dimethylsulfide [DMS]). The periplasmic nitrate reductase NapABCGH is produced under nitrate-limiting conditions and is also believed to be involved in redox balancing. FdhF is part of the formate/hydrogen lyase system oxidizing formate and producing hydrogen. The cofactors of the Hyc-subunits are not shown. BisC is the cytoplasmic biotin sulfoxide reductase. (B) Localization of enzymes of the xanthine oxidase family. PaoABC is localized in the periplasm and detoxifies aromatic aldehydes. XdhABC is the cytoplasmic xanthine dehydrogenase using NAD^+ as terminal electron acceptor. The role of XdhD is so far unclear. (C) Localization of enzymes of the sulfite oxidase family. So far, YedY is the only characterized member of the sulfite oxidase family in *E. coli*. YedY is located in the periplasm and forms a complex with YedZ. The physiological substrates for YedY, binding the Mo-MPT-form of Moco, are not known to date.

YdhV shares homologies to the aldehyde ferredoxin oxidoreductase from *P. furiosis*, binding tungsten-containing bis-MPT. The protein belongs to the *ydhYVWXUT* operon with *ydhY* and *ydhX* coding for a 4Fe4S ferredoxin-like protein, *ydhV* for the oxidoreductase subunit, *ydhU* for a cytochrome and *ydhW* and *ydhT* for proteins with unknown function. An *ydhV* mutant is impaired in the anaerobic utilization of certain sulfur sources (L-cysteine, D-cysteine, L-cysteic acid, hypotaurine and butane sulphonic acid) [62]. Further characterizations of the protein have not been reported so far.

Additionally, YcbX and YiiM were identified to be involved in the Moco-dependent pathway for detoxification of N-hydroxylated base analogs, in particular in the oxidation of 6-N hydroxylaminopurine (HAP) (Table 1) [63]. YcbX contains an N-terminal β-barrel domain, a central MOSC-domain and a C-terminal 2Fe2S ferredoxin-like domain. YiiM contains an N-terminal MOSC domain and a C-terminal helical domain. MOSC family proteins are described to bind the molybdenum cofactor [64]. Likely, these proteins belong to the sulfite oxidase family of molybdoenzymes, since they contain a conserved cysteine at their active site. It has not been determined yet whether YiiM and YcbX bind the Mo-MPT form of Moco or a dinucleotide variant.

5. Biosynthesis of the molybdenum cofactor

Much has been learned about Moco biosynthesis (Fig. 3) from studies of Moco mutants in *E. coli* where six loci have been implicated in the pleiotrophy of the molybdo-enzymes: *moa*, *mob*, *moc*, *mod*, *moe*, and *mog*, comprising 16 genes (Fig. 4) [65,66]. With the exception of *mod* encoding the genes for the high affinity molybdate transport system, all of these are involved in the biosynthesis of Moco. The biosynthesis of Moco can be divided into four steps in *E. coli* (Fig. 3) [10]: (1) formation of cyclic pyranopterin monophosphate (cPMP), (2) formation of MPT from cPMP, (3) insertion of molybdenum to form Moco, and (4) additional modification of Moco with the attachment of GMP or CMP, forming the MPT-guanine dinucleotide cofactor (MGD) or the MPT-cytosine dinucleotide cofactor (MCD). Each of these steps will be described in detail below.

5.1. Conversion of GTP to cyclic pyranopterin monophosphate (cPMP)

In *E. coli*, the *moaA* and *moaC* gene products are responsible for the complicated chemical reactions required to generate cPMP. Labeling

studies had determined that a guanosine derivative was the initial starting point for cPMP formation [67,68]. In an in vitro system for cPMP synthesis containing the MoaA and MoaC proteins purified from *Staphylococcus aureus* it was demonstrated that 5′-GTP is the specific initial substrate for Moco biosynthesis [69]. As shown in Fig. 5, formation of cPMP from GTP involves several steps including opening of the guanine imidazole ring to generate a formyl-diaminopyrimidine nucleotide intermediate, insertion of the guanine C-8 formyl group between C-2′ and C-3′ of the ribose, closure of the new pterin ring, generation of cyclic monophosphate with elimination of pyrophosphate, and formation of a pyran ring through attack by the 3′ hydroxyl of the side chain on the pterin. The cooperative participation of the MoaA and MoaC proteins in this step of Moco biosynthesis brings about significant carbon rearrangements whose net result is that the two carbon atoms added into the newly formed cPMP pterin ring originated as C-1′ and C-2′ of the GTP ribose ring [70,71], and the 4-carbon side chain is formed from the GTP guanine C-8 and C-3′, and C-4′ and C-5′ of the GTP ribose in that order [67,68].

MoaA has been classified as a member of the S-adenosylmethionine (SAM)-dependent radical enzyme superfamily [72]. In the reactions catalyzed by members of this family, SAM serves as the free radical initiator and undergoes cleavage to methionine and a 5′deoxyadenosyl radical that in turn initiates radical formation of substrate molecules or of glycyl residues within the target enzymes to activate them for radical-based chemistry. The source of the electron required for the cleavage of SAM is a reduced form of a conserved FeS cluster within the protein [72]. MoaA contains two oxygen-sensitive [4Fe-4S] clusters, one typical for SAM-dependent radical enzymes at the N-terminus and an additional C-terminal cluster unique to MoaA proteins [69]. The 5′ desoxyadenosyl radical generated from SAM at the N-terminal cluster was proposed to be in close proximity to the 5′-GTP where it is probably involved in facilitating hydrogen abstraction at either the C8 of the guanine or the C2′ or C3′ atoms of the ribose [71]. Insertion of the formyl group between the ribose C2′ and C3′ carbons might also require radical mediation. The proposed intermediates during the reaction catalyzed by MoaA are shown in Fig. 5.

The role of MoaC in the reaction is not completely clear yet; however, it has been speculated that MoaC is involved in the cleavage of the dihydropyrazine-type intermediate pyrophosphate group and formation of the cPMP cyclic phosphate group [73].

5.2. Insertion of sulfur and formation of MPT

While cPMP is structurally quite similar to MPT, it lacks the dithiolene function essential for molybdenum ligation in Moco. Therefore, for the conversion of cPMP to MPT, two sulfur atoms must be incorporated at the C1′ and C2′ positions of cPMP (Figs. 3 and 6). In *E. coli* this reaction is catalyzed by the MPT synthase protein [74]. Purification of this MPT synthase activity identified a heterotetrameric enzyme consisting of two small (~8,750 Da) and two larger (~16,850 Da) protein subunits, which are encoded by the *E. coli moaD* and *moaE* loci, respectively [75,76]. MoaD carries the sulfur used for generation of Moco in form of a C-terminal thiocarboxylate group, while MoaE binds the cPMP substrate [77,78].

A model has been proposed for the insertion of the two sulfur atoms into cPMP catalyzed by MPT synthase (Fig. 6B): Initial attack and transfer of the first sulfur atom from a MoaD thiocarboxylate occurs at the C2′ position and is coupled to hydrolysis of the cPMP cyclic phosphate [78]. During the course of this reaction, an intermediate is formed in which the MoaD C-terminus is covalently linked to the

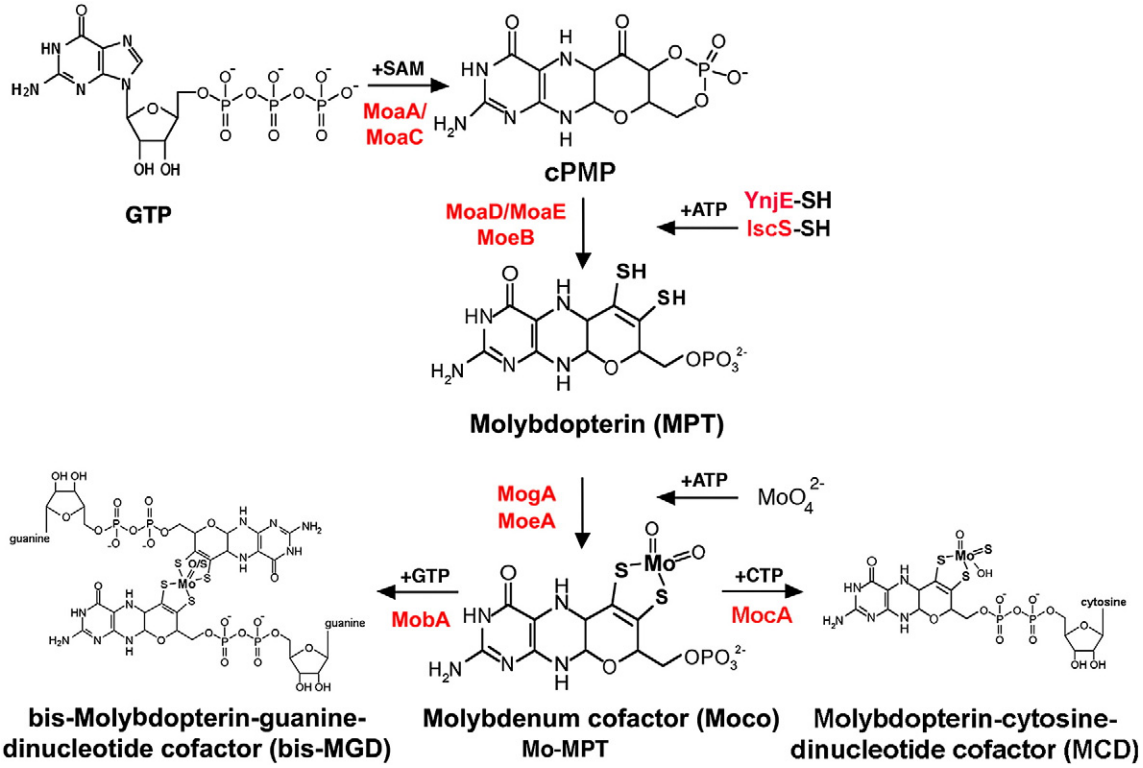

Fig. 3. The biosynthesis of Moco in *E. coli*. Shown is a scheme of the biosynthetic pathway for Moco biosynthesis in *E. coli* and the proteins involved in this pathway. Moco is formed from 5′GTP via the cPMP and MPT as intermediates. For enzymes of the DMSO reductase family, Moco is further modified by the attachment of GMP to form MGD, and two equivalents of MGD are bound to a single molybdenum to form the bis-MGD variant of the cofactor. Additional ligands can be an oxo- or a sulfido group in addition to a serine, a cysteine, a selenocysteine or a hydroxide and/or water molecule. For enzymes of the sulfite oxidase family, Mo-MPT is directly inserted without further addition of a nucleotide and coordinated by an additional cysteine ligand in the enzyme. For enzymes of the xanthine oxidase family in *E. coli*, Moco is further modified by the addition of a cytosine nucleotide to form the MPT-cytosine dinucleotide cofactor (MCD). Additionally, a terminal sulfur ligand is added to the molybdenum site, generating sulfurated MCD. An additional ligand at the Mo-center usually is a hydroxo-group. The names of the proteins involved in the reactions are colored in red.

substrate via a thioester linkage, which subsequently is hydrolyzed by a water molecule. After transfer of its thiocarboxylate sulfur to cPMP, the first MoaD subunit dissociates from the MPT synthase complex [78,79]. This would be followed by subsequent binding of a second, thiocarboxylated MoaD to the active site and transfer of the second sulfur to form MPT. In the course of the reaction of the first sulfur transfer, the opening of the cyclic phosphate is proposed to shift the location of the intermediate within the protein so that the C1′ position now becomes more accessible to attack by the second MoaD thiocarboxylate. This results in a second covalent intermediate that is converted to MPT via the elimination of a water molecule and hydrolysis of the thioester intermediate. In this model, each cPMP molecule remains bound at a single active site until conversion to MPT is completed by the exchange of carboxylated and thiocarboxylated MoaD molecules.

For MPT synthase to act catalytically, it is necessary to regenerate the transferable sulfurs at the C-terminus of the MoaD proteins after the sulfurtransfer to cPMP [76], a reaction catalyzed by the MoeB protein (Fig. 6A). It was demonstrated that MoeB-dependent activation of MoaD required ATP consumption [80]. The first step in this activation process is the formation of an acyl-adenylate intermediate at the C-terminal MoaD glycine. After the formation of the activated MoaD acyl-adenylate in the complex with MoeB, sulfur is inserted to form the terminal MoaD-thiocarboxylate group on MoaD-Gly81. It was determined that in *E. coli*, L-cysteine serves as the origin of the MPT dithiolene sulfurs and that the cysteine sulfur is transferred to the activated MoaD acyl-adenylate by the action of a persulfide-containing protein [81]. Further investigations showed that this protein is the pyridoxal-phosphate-dependent *E. coli* L-cysteine desulfurases IscS [82]. Its involvement in Moco biosynthesis added another biosynthetic pathway to the versatile role of IscS, acting as sulfur donor for the biosynthesis of FeS clusters, biotin, thiamine, lipoic acid, and sulfur-containing bases in tRNA [83,84]. However, recent studies showed that the rhodanese-like protein YnjE acts additionally in the sulfurtransfer reaction, directing IscS towards Moco biosynthesis [85]. Likely, IscS has several interaction partners, but the interaction does not occur directly with the partner protein, since mediator proteins were identified. These proteins which act as mediators with IscS are in thiamine biosynthesis the ThiI protein, transferring sulfur to ThiS, the IscU protein in FeS cluster biosynthesis, transferring the cluster to IscA or the target protein, or TusA in tRNA thiolation, transferring the sulfur to TusBCD, TusE, MnmA and then to tRNA [84]. The mediator protein in Moco biosynthesis is the rhodanese-like protein YnjE.

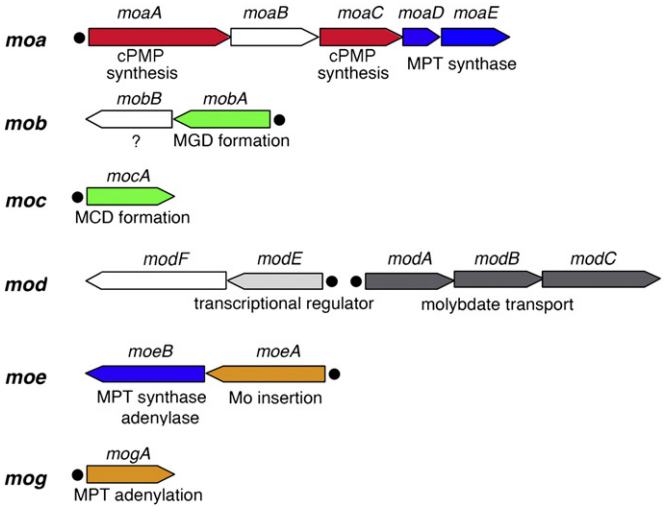

Fig. 4. Organization of the genes involved in the biosynthesis of Moco in *E. coli*. In total, 16 genes are involved in the biosynthesis of Moco in *E. coli*. These are organized into 6 different gene loci termed *moa*, *mob*, *moc*, *mod*, *moe* and *mog*. Genes of known function are colored: grey, Mo-transport; red, cPMP biosynthesis; blue, MPT synthesis; orange, Mo-ligation; green, nucleotide addition. Black dots indicate promotor regions. The genes are drawn approximately to scale. Additional operons involved in Moco biosynthesis, such as the *isc* operon, which is also involved in other biosynthetic pathways, are not shown.

Summarizing the results, a sulfurtransfer mechanism for the formation of the MoaD-thiocarboxylate group is proposed (Fig. 6A) [85,86]. In the $(MoaD–MoeB)_2$ complex, MoeB activates MoaD by formation of an acyl-adenylate bond, and IscS in its persulfide-bound form is able to interact with this complex in addition to YnjE. A possible reaction could be that MoaD is transferred to the YnjE-C385 persulfide group, forming a disulfide bond. Release of the MoaD thiocarboxylate requires a second thiol group, which is proposed to be MoeB-Cys187, but other reducing factors are also possible. It is proposed that all proteins form a complex, making the sulfur transfer more specific.

After the reaction, MoaD-SH dissociates from the complex, and reassociates with MoaE to form active MPT synthase (Fig. 6). The binding constants within the different complexes of MoaD were shown to follow the order $(MoaD–SH–MoaE)_2 > (MoaD–MoeB)_2 > (MoaD–MoaE)_2$ [87]. This order is mechanistically logical given that during the course of MPT biosynthesis, MoaD-SH first binds to MoaE to form the active MPT synthase complex where transfer of the MoaD-SH thiocarboxylate to cPMP occurs, yielding MPT and inactive MPT synthase. MoaD must then dissociate from this inactive complex to form a new complex with MoeB, a prerequisite for the regeneration of MoaD-SH. In addition, the $(MoaD–MoeB)_2$ complex is stabilized by ATP addition and the subsequent formation of the acyl-adenylate on MoaD. In this form, IscS/YnjE-SH transfer the sulfur to MoaD, generating MoaD-SH. After the formation of the $(MoaD–SH–MoaE)_2$ complex, introduction of the dithiolene moiety in MPT completes the formation of the chemical backbone necessary for binding and coordination of the molybdenum atom in Moco (Fig. 3).

5.3. Insertion of molybdenum into MPT

In *E. coli*, insertion of the metal into the MPT is accomplished by the *moeA* and *mogA* gene products (Figs. 2 and 7) [88,89]. The structure of *E. coli* MogA was the first Moco biosynthesis protein crystal structure to be reported in 2000 [90]. MogA is a trimer in solution with each monomer folded into a single compact domain, and MogA binds MPT with high affinity [90]. The crystal structures for *E. coli* MoeA showed a dimeric structure with an elongated monomer consisting of four distinct domains, one of which was structurally related to MogA [91,92]. It was shown that the proteins have different functions in the molybdenum chelation reaction (Fig. 7) [93]. MoeA appeared to mediate molybdenum ligation to newly synthesized MPT in vitro at low concentrations of MoO_4^{2-}. This reaction was strongly inhibited by MogA in the absence of ATP, but in the presence of ATP, MogA doubled the rate of molybdenum ligation [93]. Later, the catalytic formation of an MPT–AMP intermediate during the reaction was shown [94–96], which was identified by the crystal structure of Cnx1 to accumulate on a variant of the *Arabidopsis thaliana* Cnx1-S583A protein [96]. The accumulation of a comparable MPT–AMP intermediate in *E. coli moeA*$^-$ extracts could be verified (unpublished data). In *E. coli* MPT is activated by MogA-mediated adenylation prior to molybdenum insertion by MoeA under physiological molybdenum concentrations (Fig. 7). However, this reaction is not absolutely required under high molybdenum concentrations, since *mogA*$^-$ cells were rescued for molybdoenzyme activities by the addition of high molybdate concentrations to the medium [97].

It was shown that under physiological molybdate concentrations (1–10 μM), MogA is required in *E. coli* to form an MPT–AMP intermediate that facilitates molybdate insertion on the dithiolene sulfurs. Under high molybdate concentrations (>1 mM), MPT–AMP formation by MogA is not required and molybdate can be directly inserted into MPT with the aid of the MoeA protein [98]. Additionally, it was shown that bivalent copper and cadmium ions as well as trivalent

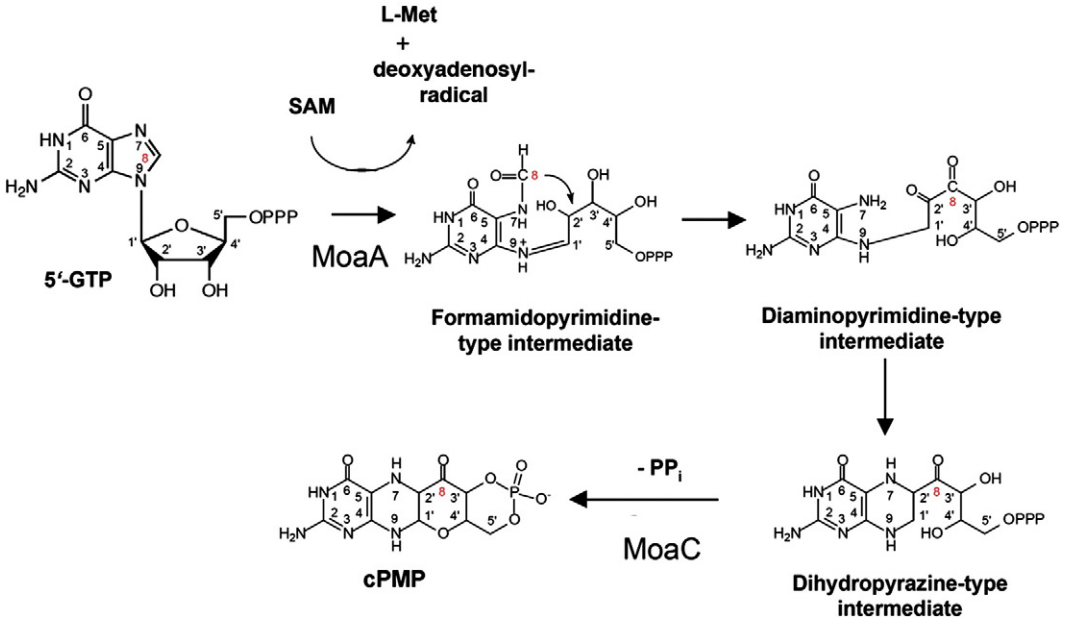

Fig. 5. The biosynthesis of cPMP from 5′GTP. All the carbon and nitrogen atoms of the initial GTP molecule are retained within cPMP during synthesis, and the numbering system matches that of the GTP carbons through all structures shown. The C8 atom of the guanine ring is transferred as a formyl group and is inserted between the C2′ and C3′ atoms of the ribose. Hypothetical formamidopyrimidine-type, diaminopyrimidine-type and dihydropyrazine-type intermediates that might be formed during the reaction are shown. This reaction is catalyzed by the MoaA protein, a S-adenosylmethionine(SAM)-dependent enzyme. MoaC is believed to cleave the pyrophosphate group of the dihydropyrazine intermediate. cPMP is shown in the dihydropyrano form.

arsenite ions could all insert nonspecifically into MPT without the presence of either MoeA or MogA and that copper had a higher affinity for the dithiolene group of MPT than molybdate [98]. Thus, bivalent metal ions in high concentrations might inhibit Moco biosynthesis in *E. coli*.

5.4. Additional modification of Moco by attachment of GMP and CMP

The proteins of the xanthine oxidase and DMSO reductase family in *E. coli* contain the dinucleotide derivatives MCD and MGD of the molybdenum cofactor, respectively. In *E. coli*, most enzymes belong to the DMSO reductase family of molybdoenzymes binding bis-MGD [6]. MGD is formed by covalent addition of the GMP moiety from GTP to the C4′ phosphate of MPT via a pyrophosphate bond leading to release of the β- and γ-phosphates of GTP as pyrophosphate (Fig. 8) [99,100]. This reaction is catalyzed by MobA, one of two proteins encoded by the *mob* locus (Fig. 4) [101]. While MobA has been shown to be essential for this reaction [102], the role of MobB, the second protein encoded by the *mob* locus, remains uncertain. Based on its crystal structure, it was postulated that MobB could be an adapter protein, acting in concert with MobA to achieve the efficient biosynthesis and utilization of MGD [103]. In vitro, however, MobA, GTP, $MgCl_2$ and Mo-MPT are sufficient for the formation and insertion of bis-MGD into *R. sphaeroides* DMSO reductase without MobB [100,104]. It was shown that the addition of the nucleotide to the cofactor occurs only after insertion of molybdenum into MPT [105]. The crystal structure of monomeric MobA showed a nucleotide-binding Rossman fold formed by the N-terminal half of the protein that also contains the GTP binding site. A possible MPT binding site was localized to the C-terminal half of the protein [100,106].

More recently, three enzymes were identified in *E. coli* belonging to the xanthine oxidase family (XdhABC, XdhD, and PaoABC) which bind the MCD form of the cofactor (Table 1, Fig. 2) [107,108]. MCD formation is catalyzed by a protein which was named MocA, for molybdopterin cytosine dinucleotide synthesis (Fig. 4). MocA was identified by amino acid sequence comparison to MobA, since they exhibits 22% amino acid sequence identity [109]. The catalytic reaction of MocA is similar to the reaction of MobA, in that it acts as a CTP:molybdopterin cytidylyl transferase and covalently links MPT and CMP with the concomitant release of the β- and γ-phosphates of CTP as pyrophosphate (Fig. 8) [109].

Comparison of the two transferases revealed that MobA is highly specific for binding of the purine nucleotide GTP, while MocA is specific for binding of the pyrimidine nucleotide CTP [110]. The most significant sequence differences between the two proteins were observed in two conserved motifs at their N-terminal domain. The crystal structure of MobA with bound GTP showed that the guanine moiety is mainly bound by the ^{12}LAGG^{15} and ^{78}GPLAG^{82} amino acid sequence segments [100,111]. In MocA, these sequences are altered to ^{12}TAAG^{15} and ^{78}GLLTS^{82}. Site directed mutagenesis studies revealed that the introduction of only 5 amino acid exchanges in the two N-terminal regions of either MobA or MocA was sufficient to cause loss of specificity for the pyrimidine or purine nucleotides so that both proteins were able to bind either CTP or GTP to almost the same extent. In addition, the C-terminal domains of MocA and MobA have been found to play an important role in determining the specificity of their interaction with the target molybdoenzymes [110]. This domain is involved in MPT binding [111].

6. Further modification of Moco

6.1. Sulfuration of MCD and maturation of Moco for enzymes of the xanthine oxidase family of molybdoenzymes

Apart from the attachment of additional nucleotides in prokaryotes, Moco can be further modified by an exchange of the equatorial molybdenum oxygen ligand with a sulfur atom (Figs. 3 and 8). Sulfurated Moco is the characteristic form of the cofactor present in all members of the XO family [6]. Cofactor sulfuration is mediated by the help of members of the XdhC family of molecular chaperones. These molecular chaperones are required for the maturation of molybdoenzymes of the xanthine oxidase family but are not part of the active holo-molybdoenzymes themselves [112]. The best characterized chaperone from this family is the *Rhodobacter capsulatus* XdhC protein [37]. Investigation of *R. capsulatus* XdhC showed that

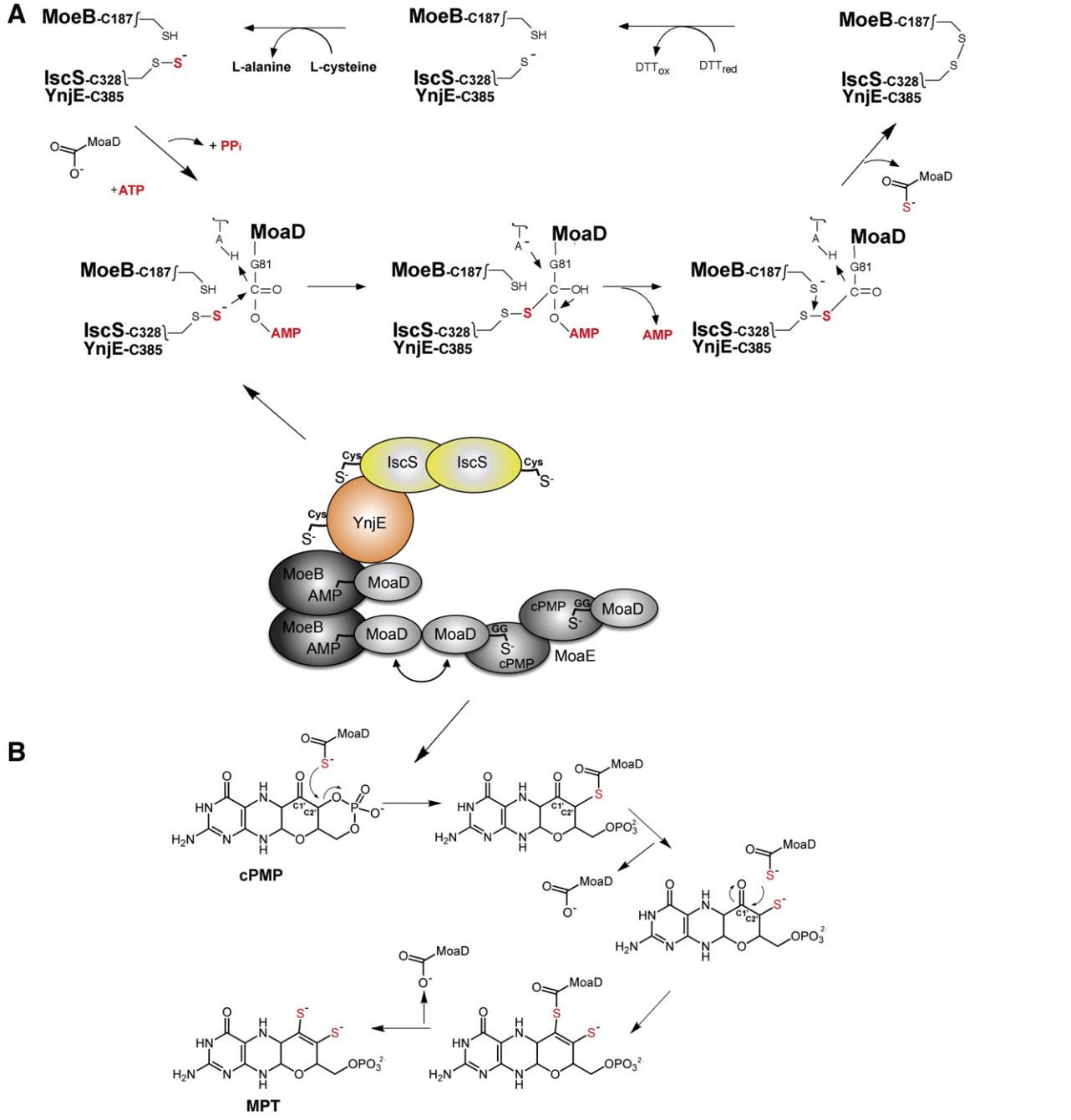

Fig. 6. The biosynthesis of MPT from cPMP. cPMP is converted to MPT by the transfer of two sulfur groups from the C-terminal thiocarboxylate of the MoaD subunit of MPT synthase. (A) Formation of the thiocarboxylate group on *E. coli* MoaD. Regeneration of the MoaD thiocarboxylate sulfur occurs in a MoaD/MoeB complex where adenylated MoaD is formed by attachment of an AMP moiety at the MoaD C-terminus with loss of pyrophosphate. MoaD-AMP is then sulfurated by a protein-bound persulfide group on the IscS/YnjE sulfurtransferases, likely by the formation of an YnjE-MoaD disulfide intermediate. Reductive cleavage of the disulfide bond could then occur by attack of the thiol group of MoeB-C187, and in turn, thiocarboxylated MoaD is generated and released from the ternary complex. The disulfide bond formed between MoeB and YnjE is likely reduced by the tioredoxin system in vivo (in vitro reduction by DTT is shown). The sulfur donor for IscS is L-cysteine. After formation of the thiocarboxylate group, MoaD dissociates from the MoeB dimer and reassociates with MoaE. The proteins are expected to form a complex in the cell. (B) In the MPT synthase mechanism, the initial attack and transfer of the first MoaD-SH sulfur atom occurs at the C2′ position, coupled to the hydrolysis of the cPMP cyclic phosphate. An intermediate is formed in which the MoaD C-terminus is covalently linked to the substrate via a thioester linkage that is subsequently hydrolyzed by a water molecule and generates a hemisulfurated intermediate at C2′. Opening of the cyclic phosphate shifts the location of the intermediate within the MoaD to a position where C1′ becomes more accessible. A new MoaD thiocarboxylate attacks the C1′ resulting again in a second covalent intermediate which is converted to MPT via the elimination of a water molecule and hydrolysis of the thioester intermediate. On the left side, the structure of cPMP is shown with the C1′ and C2′ carbons labeled.

it binds the Moco produced by MoeA/MogA and protects it from oxidation until the terminal molybdenum sulfur ligand is inserted [105,113]. XdhC also interacts with the *R. capsulatus* L-cysteine desulfurase, NifS4, the protein that actually replaces the cofactor equatorial oxygen ligand with a sulfido ligand [114]. The sulfur for this reaction originates from L-cysteine, and a NifS4 persulfide group is formed during the course of the reaction. After the sulfuration reaction, it is believed that XdhC with its bound sulfurated Moco dissociates from NifS4 and forms a new interaction with the XdhB subunits of the *R. capsulatus* $(\alpha\beta)_2$ XDH heterotetramer [105,115].

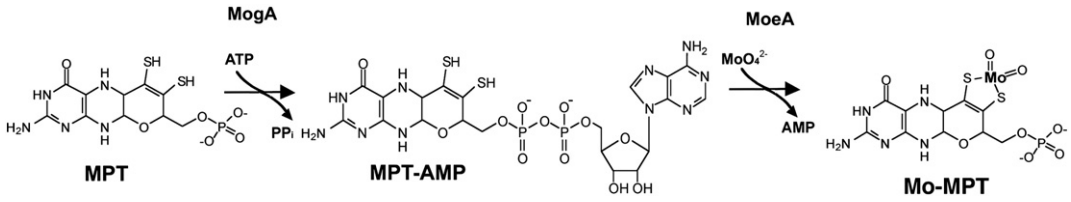

Fig. 7. Insertion of molybdate into MPT. The *E. coli* MoeA and MogA proteins catalyze the specific incorporation of molybdenum into MPT in a multistep reaction with an adenylated MPT intermediate (MPT–AMP). While MogA forms the MPT-adenylate intermediate, MoeA mediates molybdenum ligation to MPT at low concentrations of MoO_4^{2-}.

Thus, it appears from the *R. capsulatus* studies that XdhC-like proteins perform a number of functions including stabilization of the newly formed Moco, interaction with an L-cysteine desulfurase to ensure that Moco sulfuration occurs [114] as well as interaction with their specific target proteins for insertion of the sulfurated Moco [37]. Because Moco is deeply buried in the protein, it is also believed that the XdhC proteins may act as chaperones to facilitate the proper folding of the target proteins after Moco insertion [112]. This model implies that molybdoenzymes requiring the sulfurated form of Moco exist in a Moco competent "open" apo-molybdoenzyme conformation until the

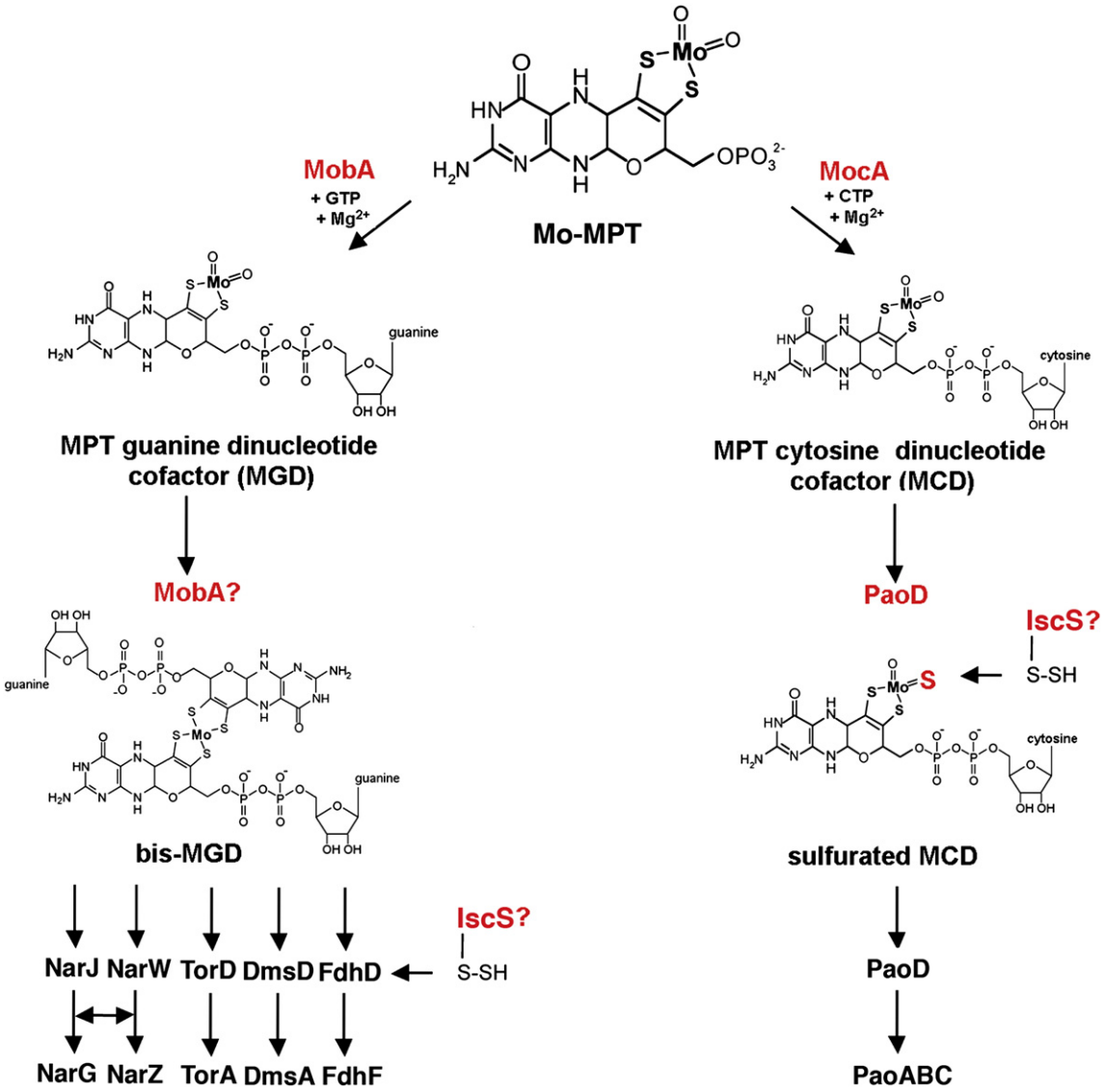

Fig. 8. Molybdopterin dinucleotide biosynthesis. Synthesized Moco can be further modified in *E. coli* by the addition of GMP or CMP to the C4′ phosphate of MPT via a pyrophosphate bond. MGD is formed by the MobA protein, which specifically binds GTP, while MCD is formed by the MocA protein, which acts specifically on CTP. In both cases, it has been shown that addition of the dinucleotide to the cofactor occurs after insertion of molybdenum into MPT. In all MGD-containing molybdoenzymes in *E. coli*, the molybdenum atom is coordinated by the dithiolene groups of two MGD molecules, forming the bis-MGD cofactor. The protein involved in this reaction has not been identified yet; however, this step may be catalyzed by MobA. MGD-binding chaperones were described for severel molybdoenzymes. NarW and NarJ were shown to be interchangeable. FdhD was shown to be involved in the sulfuration of MGD (catalyzed by IscS) for FdhF. For PaoABC, MCD is further modified in *E. coli* by exchange of the equatorial oxygen to a sulfido ligand, forming sulfurated MCD. This step is carried out by the PaoD protein in conjunction with an as yet unidentified sulfurtransferase (likely IscS). After sulfuration, MCD is inserted by the aid of PaoD into PaoABC.

insertion of sulfurated Moco. After insertion, the protein adapts the final active "closed" conformation that can no longer accept Moco [112].

The *E. coli* aldehyde-oxidoreductase PaoABC protein complex has been shown to contain the sulfurated MCD cofactor [38], and the PaoD protein was shown to be essential for the insertion of sulfurated MCD into PaoABC (Fig. 8, unpublished results). PaoD is the last protein encoded by the *paoABCD* operon in *E. coli*. Since PaoD belongs to the XdhC family, it is expected to play a role similar to that of XdhC with the only difference being that PaoD facilitates sulfuration and insertion of an MCD cofactor rather than an MPT cofactor (Fig. 8) [37]. The specific L-cysteine desulfurase involved in the sulfuration of PaoD-bound MCD has not been identified in *E. coli* to date, but it is expected that IscS performs this role in *E. coli*. The only other XdhC-like protein that is present in *E. coli* is the YqeB protein. YqeB is expected to play a similar and shared role for both XdhABC and XdhD, since it is located in the vicinity of both gene regions in the *E. coli* genome [37]. YqeB is a little larger in size than other members of the XdhC family and it contains an NAD(P) binding Rossman fold. No further data are available on YqeB.

6.2. Maturation of Moco for the DMSO reductase family of molybdoenzymes

6.2.1. Bis-MGD formation

As mentioned earlier, in all *E. coli* MGD-containing molybdoenzymes, a single molybdenum atom is coordinated by the dithiolene groups of two MGD molecules, forming the bis-MGD cofactor [116]. The structure of bis-MGD Moco was verified by the crystal structure of DMSO reductase from *R. sphaeroides* by Schindelin et al. [33]. The formation of bis-MGD is one of the most enigmatic steps in *E. coli* Moco biosynthesis. It is still not known whether the two MGD molecules assemble on MobA or at the cofactor binding sites of the molybdoenzymes themselves (Fig. 8). Crystal structures for both monomeric and octameric forms of MobA have been reported [100]. While a binding groove that could accomodate MGD was identified in the monomer, there was no comparable position in the monomer large enough for bis-MGD binding. However, the central cavity of the octameric form of MobA is large enough to support bis-MGD formation, and hydrophilic residues in the octamer channel could facilitate bis-MGD binding and storage prior to insertion into molybdoenzymes [100]. Alternatively, since MGD binding to proteins involved in the maturation of several molybdoenzymes such as TorD protein, the system-specific chaperone for *E. coli* TMAO reductase, has been observed, it is also possible that the bis-form of the MGD cofactor is formed in conjunction with these types of proteins [117].

6.2.2. Sulfuration of bis-MGD

E. coli expresses three FDHs that decompose formate to carbon dioxide and protons. FdnGHI and FdoGHI are respiratory enzymes anchored to the periplasmic side of the inner membrane [53]. FdhF is part of the fermentative formate-hydrogen lyase complex and is located at the cytoplasmic side of the membrane [118]. FdhF belongs to the DMSO reductase family of molybdoenzymes containing a bis-MGD cofactor [6]. Additionally, these enzymes contain selenocysteine at the active site, functioning as a ligand to the Mo-site. Reinterpretation of the original crystal data of FdhF suggested that at the molybdenum site, the apical ligand is rather a sulfur ligand instead of an oxygen ligand [44]. Thus, in the oxidized state, the enzyme contains the four pterin sulfur ligands at the Mo site, a selenocysteine ligand and a –SH ligand. The chaperone involved in sulfuration of the Moco for FdhF was shown to be the FdhD protein [119]. FdhD was proposed to bind Moco (likely bis-MGD); thus, bis-MGD might be directly sulfurated on FdhD. Additionally, FdhD interacts with IscS, which allows transfer of sulfur from persulfide-IscS to form a new persulfide group on a conserved cysteine on FdhD. Finally, sulfurated bis-MGD might be transferred to FdhF. The additional sulfur atom at the active site of FdhF subsequently produces an active enzyme [119].

In addition to FDHs, the DMSO reductase family includes other members for which an additional sulfur ligand of the molybdenum atom has been reported at the catalytic site. The x-ray crystal structure of the periplasmic nitrate reductase (Nap) of *Cupriavidus necator* showed the presence of a terminal sulfur ligand at the molybdenum coordination sphere [120]. Similar data were obtained for the homologous NapA protein from *Desulfovibrio desulfuricans* ATCC 27774, for which the crystal structure showed a unique coordination sphere of six sulfur ligands bound to the molybdenum atom [121]. Conclusively, these observations might suggest that sulfuration of bis-MGD is more common of this group of enzymes than previously expected.

7. Insertion of bis-MGD into molybdoenzymes of the DMSO reductase family and the role of molecular chaperones

The majority of the molybdoenzymes in *E. coli* belong to the DMSO reductase family that contain the bis-MGD cofatctor (Table 1). Generally, these enzymes are either associated with the inner membrane or are located in the periplasm (Fig. 2). The insertion of the molybdenum cofactor into these enzymes is a cytoplasmic event required before the translocation of periplasmic molybdoproteins by the Tat-machinery [45,46]. For the insertion of bis-MGD, molecular chaperones have been described, which act specific with their respective molybdoenzyme partner for its maturation [116]. The best described system is the TorA–TorD system in *E. coli*. However, molecular chaperones have been also described for other systems: NarJ is the chaperone for nitrate reductase A NarGHI [122], NarW is the chaperone for nitrate reductase Z NarZYV [123], and these chaperones were shown to act interchangeable, DmsD is the chaperone for DmsABC [124], and YnfE/F [59] and FdhD is the chaperone for FdhF (Table 1) [119]. Here, we will describe the TorD family of molecular chaperones in detail in addition to its mechanism of action for the maturation of TorA [116]. Whether the mechanism of TorD is a general maturation system that can be applied to all other molybdoenzymes of the DMSO reductase family remains to be determined.

7.1. The TorD family of molecular chaperones

The TorD family of molecular chaperones contains hundreds of members that are mainly bacterial but also a few archaeal proteins. Although these proteins present a low level of sequence identity (20% or less), the members of this family are characterized by a highly conserved "E(Q)PxDH" motif [117,125]. The crystal structures of TorD protein members showed that they generally contain 10–12 α-helices that account for 60–70% of the protein residues. They contain in addition a long loop region separating the N- and C-terminal domains of the proteins and holding the "E(Q)PxDH" highly conserved motif [126,127].

For TorD was shown that it interacts with its molybdoenzyme partner TorA at least with two distinct regions: one of the binding sites is located near the N-terminal extremity of the catalytic protein while the other is in the core [128–130]. TorD recognizes the Tat-leader peptide of TorA; however, it was shown that the twin-arginine motif is not necessary for this interaction and that only the h- and c-regions of the leader peptide are recognized by TorD [130,131]. This interaction is specific, since other Tat signal sequences like those of *E. coli* HybO or even closer TorA ortholog (for instance TorA from *S. oneidensis*) were not recognized by *E. coli* TorD [131].

The additional interaction with the amino-terminal part of the enzyme is still not clearly defined. Combining random and directed mutagenesis on *dmsD*, a TorD homolog, a hot pocket of residues important for leader binding was modelled [132]. It comprises residues from helix 4 and to a lesser extent residues D and H from the conserved motif. It thus appears that the hinge region connecting the two parts

of the chaperones may have a role in the leader peptide binding. It is believed that a large area of the chaperone surface is probably involved in the leader binding [115].

The second interaction site was observed between the chaperone and the core of the molybdoenzyme [128]. By random and directed mutagenesis, it was established that helix 5 of TorD is the region involved in the TorA core binding and that helix 5 TorD variants still bind the signal sequence of TorA in vitro [115]. Since this region is not conserved in the TorD family members, it could contribute to the specificity of the chaperone and its molydboenzyme partner.

7.2. First step in TorA biogenesis: TorA maturation involves TorD protection from proteolysis

In the absence of TorD, TorA stability is drastically affected under conditions of thermal stress, molybdenum cofactor deficiency or molybdenum starvation and in optimal conditions, the absence of TorD leads also to a 70–80% decrease of TorA amount [128,133–135]. It was shown that TorD stabilizes the TorA signal sequence from degradation (Fig. 9), implying that TorD binds to the signal sequence of TorA right after its release from the ribosome. In the absence of TorD, TorA is proteolized by a two-step mechanism: most of the signal sequence is first cleaved and the remaining of the protein is subsequently rapidly degraded [135]. Thus the first role of TorD is to protect the TorA signal sequence from degradation which then allows the interaction with the translocation machinery and its transport to the periplasm [135]. Interestingly, it was shown that other members of the TorD family also interact with the N-terminal region of their partner. It is the case for NarJ that binds the N-terminal extremity of NarG containing a proposed remnant signal sequence [136]. DmsD is another exemple since it interacts with the leader peptide of DmsA subunit of the DMSO reductase [124,137–139].

7.3. Second step: TorA maturation requires TorD chaperone activity

In an in vitro approach it was shown that the maturation level of apoTorA with or without its signal peptide is similar. This indicates that the sole binding to the core of the apoprotein by helix 5 of TorD drives the maturation process (Fig. 9) [125,140].

Additionally, it was shown that TorD also plays a direct role in the insertion of the molybdenum cofactor [115]. Purified apoTorA mixed with a source of molybdenum cofactor was matured in vitro to a basal level corresponding to 20%, while in the presence of TorD the maturation of apoTorA was 80% completed [129]. The last step of molybdenum cofactor biosynthesis is driven by the MobA enzyme that catalyzes the conversion of Mo-MPT to MGD by adding a GMP group from a GTP [141]. During this reaction, TorD interacts with MobA and binds both the Mo-MPT precursor form and the MGD [115]. TorD also weakly binds GTP [130]. Thus, the chaperone could act as a platform on which the last step of the molybdenum cofactor biosynthesis is completed before its delivery to the catalytic site of the apopartner (Fig. 9). One proposed role of TorD could be that it facilitates/stabilizes the conversion of bis-MGD from MGD by MobA by stabilizing the interaction between MobA and apo-TorA for bis-MGD insertion. It is obvious that the chaperone is requested for a rapid cofactor insertion to avoid a proteolytic attack and that is consistent with its role of "facilitator" of the bis-MGD maturation and insertion. Other chaperones of the TorD family present a more complex role due to the subunit composition of the partners and their distinct metal containt. Thus, the NarGHI nitrate reductase is a multimeric protein containing FeS clusters and *b*-type hemes in addition of the molybdenum cofactor. The absence of NarJ results in a global defect in metal incorporation in the complex. Although the exact function of NarJ in this process is unclear, experimental evidences showed that the chaperone drives several events leading to the metal incorporation, the subunit assembly and the proper localization of the membrane-anchored complex [34].

7.4. Last step: delivery of the premature TorA to the Tat machinery

After bis-MGD insertion into apo-TorA, mature TorA has to be targeted to the Tat machinery followed by the release of TorD (Fig. 9). This step is not well understood yet for this system, since the Tat-leader peptide needs to be exposed for the targeting to the membrane. One possibility would be that insertion of the molybdenum cofactor into the catalytic site of the enzyme modifies the affinity between the interacting region of TorA and TorD allowing thus the Tat machinery to compete for signal sequence binding. Another hypothesis is that TorD delivers matured TorA directly to the Tat machinery but so far no direct interaction was shown between TorD and the Tat proteins. However, for DmsD, a member of the TorD family, it was shown that it interacted with the signal-binding module (TatB and TatC) of the Tat machinery that contains the Tat leader peptide recognition site [142]. This step needs to be investigated further for the TorD–TorA system.

7.5. Is TorA maturation a general system that applies for all members of the DMSO reductase family?

Most of the molybdoenzymes in *E. coli* are encoded by operons also containing a gene for a chaperone of the TorD family [117,143]. Deletion of the chaperone gene generally leads to the loss or the decrease of the stability of the molybdo-counterpart, as it was shown for the NarGHI nitrate reductase and its cognate chaperone NarJ [117,144,145]. However, exceptions exist where a chaperone has not been indentified for a molybdoenzyme so far. In *E. coli*, two characterized molybdoenzymes, the cytoplasmic BisC and the periplasmic TorZ, have no defined specific chaperone [125,139]. As no orphan *torD* homologous gene has been found so far, it remains possible that TorZ maturation either does not require a specific chaperone or some chaperones may be shared between several molybdoenzymes as it was demonstrated for DmsD that is required for DmsA and also for YnfE and YnfF stability. One noteworthy example is the Nap respiratory system, which uses a sophisticated mechanism for biogenesis of the molybdoenzyme. The periplasmic nitrate reductase Nap system is found in many different organisms [146–149]. In *E. coli*, it is made up of five proteins: the NapAB dimer, NapA being the catalytic subunit and NapC that transfers electrons to the NapAB complex, and two other proteins, NapG a periplasmic protein and NapH an integral membrane protein, containing both iron–sulphur centers are also involved in the electron transfer pathway from the ubiquinol pool to NapC [150]. Reduction of nitrate occurs in the catalytic site of NapA containing the bisMGD form of the cofactor and a [4Fe4S] cluster [151]. Similar to all periplasmic molybdoenzymes, NapA is matured in the cytoplasm and its translocation depends on the Tat machinery [152]. In contrast to the Tor or the Nar respiratory system, however, no chaperone belonging to the TorD family was found in the operon encoding the Nap system [146]. It was shown, in *E. coli*, that two cytoplasmic proteins, NapD and NapF, are involved in the biosynthesis of NapA [153,154]. The deletion of *napD* or *napF* leads to deleterious effects on the mutant growth [147]. Moreover, a direct interaction was described between NapA and NapD, and NapA and NapF [154]. NapD binds the NapA Tat signal sequence and this interaction overlaps significantly with the twin arginine motif. Moreover during this interaction, studied by NMR, the signal peptide adopts a α-helical conformation that reminds the α-helical conformation of the N-terminus extremity of NarG conserved after NarJ binding [155–157]. NapD presents a ferredoxin-like fold and could operate as a quality control system for NapA translocation [148]. Interestingly, NapF was shown to transfer in vitro a [4Fe4S] cluster to NapA and was proposed to facilitate the insertion of the cofactors into NapA [153,154]. Thus, in *E. coli* biosynthesis of NapA appears to require two proteins, NapD and NapF, involved in the targeting and the maturation of NapA, respectively.

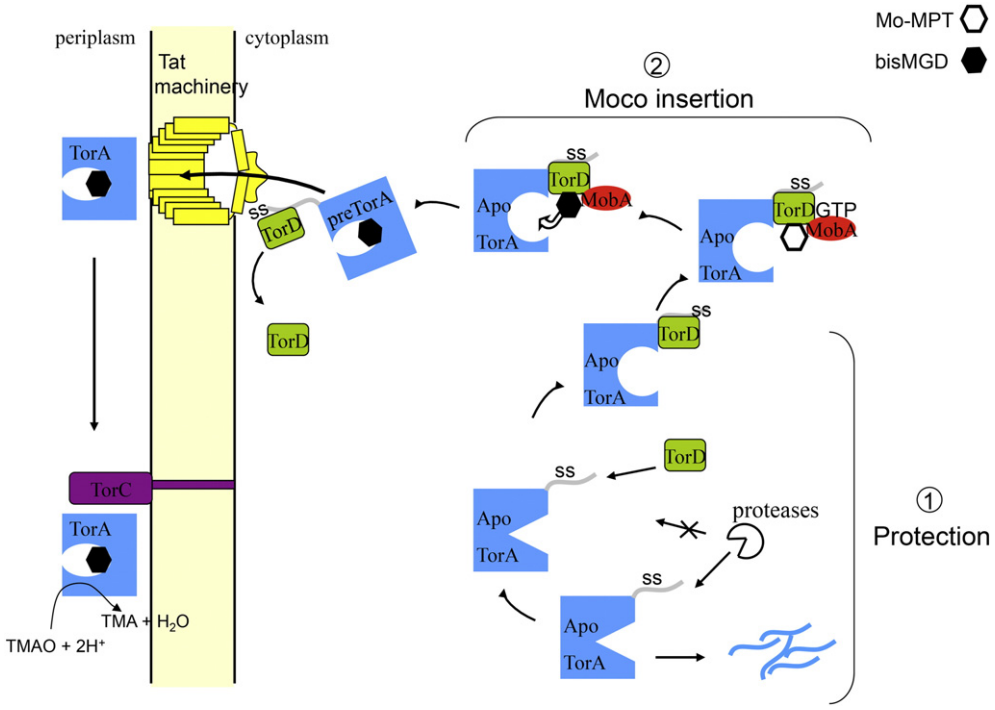

Fig. 9. Model for TorD-dependent TorA maturation. Maturation of the TorA molybdoenzyme required TorD, its specific chaperone for protection against proteolytic attack (1) and insertion of bisMGD, the mature form of the molybdenum cofactor (2). The translocation of the folded TorA is catalyzed by the Tat-machinery. ss: signal sequence.

8. Concluding remarks

More than 30 years after publication of the first proposed structure for Moco, a number of questions remain about the pathway of Moco biosynthesis. This review mainly focusses on Moco biosynthesis, molybdoenzymes and their maturation in *E. coli*. While conserved genes for Moco biosynthesis are found in bacteria, archaea, fungi, plants and animals, it has long been proposed that, with the exception of some organisms like yeast that apparently possesses no molybdoenzymes [158], the biosynthesis of Moco is conserved in all organisms. However, elucidation of the details of the pathway for Moco biosynthesis in a variety of organisms has revealed that significant differences in the biosynthetic pathways do exist [159,160]. For example, in higher eukaryotes, many individual Moco biosynthesis proteins appear to be involved in several different biosynthetic pathways and perform several roles as a consequence of gene sharing [159,160]. Although a wide range of variations on the basic Moco structure exists in bacteria, no dinucleotide forms of Moco have been identified in eukaryotes to date. One of the most enigmatic question is the formation and role of the bis-MGD cofactor. It was shown that MobA forms the MGD molecule after molybdenum insertion, but formation of the bis-form from two MGD molecules would require the release of one molybdenum atom. Additionally, bacteria contain a large variety of molybdoenzymes that catalyze specific, redox-reactions. In humans, however, only four molybdoenzymes have been identified, and a defect in Moco biosynthesis is lethal due to the loss of sulfite oxidase activity. In bacteria chaperones were identified to be involved in the maturation of their respective target proteins. The exact role of these chaperones still needs to be elucidated. But how does Moco insertion occur in eukaryotes, where these chaperones do not exist. Thus, many open questions remain that need to be elucidated in future studies.

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