

## Review Article

# The Biosynthesis of the Molybdenum Cofactor in *Escherichia coli* and Its Connection to FeS Cluster Assembly and the Thiolation of tRNA

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The thiolation of biomolecules is a complex process that involves the activation of sulfur. The L-cysteine desulfurase IscS is the main sulfur mobilizing protein in *Escherichia coli* that provides the sulfur from L-cysteine to several important biomolecules in the cell such as iron sulfur (FeS) clusters, molybdopterin (MPT), thiamine, and thionucleosides of tRNA. Various proteins mediate the transfer of sulfur from IscS to various biomolecules using different interaction partners. A direct connection between the sulfur-containing molecules FeS clusters, thiolated tRNA, and the molybdenum cofactor (Moco) has been identified. The first step of Moco biosynthesis involves the conversion of 5'GTP to cyclic pyranopterin monophosphate (cPMP), a reaction catalyzed by a FeS cluster containing protein. Formed cPMP is further converted to MPT by insertion of two sulfur atoms. The sulfur for this reaction is provided by the L-cysteine desulfurase IscS in addition to the involvement of the TusA protein. TusA is also involved in the sulfur transfer for the thiolation of tRNA. This review will describe the biosynthesis of Moco in *E. coli* in detail and dissects the sulfur transfer pathways for Moco and tRNA and their connection to FeS cluster biosynthesis.

## 1. An Introduction to the Importance of Molybdenum and Molybdoenzymes in Bacteria

Molybdenum is the only second row transition metal essential for biological systems, which is biologically available as molybdate ion [1]. Molybdenum 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 [2, 3]. The metal forms the active site of molybdoenzymes, which execute key transformations in the metabolism of nitrogen, sulfur, and carbon compounds [3]. The catalyzed reactions are in most cases oxo-transfer reactions; for example, the hydroxylation of carbon centers and the physiological role are

fundamental since the reactions include the catalysis of key steps in carbon, nitrogen, and sulfur metabolism.

There are two distinct types of molybdoenzymes: molybdenum nitrogenase has a unique molybdenum-iron-sulfur cluster, the  $[\text{Fe}_4\text{S}_3]\text{-(bridging-S)}_3\text{-[MoFe}_3\text{S}_3]$  center called FeMoco [4]. Nitrogenase catalyzes the reduction of atmospheric dinitrogen to ammonia. All other molybdoenzymes contain the molybdenum cofactor (Moco). In Moco the molybdenum atom is coordinated to a dithiolene group on the 6-alkyl side chain of a pterin called molybdopterin (MPT) [3, 5]. The chemical nature of Moco has been determined by Rajagopalan and coworkers in 1982 [5]. They postulated a structure of the cofactor consisting of a pterin derivative, with the pterin ring substituted at position 6 with a phosphorylated dihydroxybutyl side chain containing a cis-dithiolene bond (Figure 1). The sulfur atoms of the dithiolene group were proposed to coordinate the molybdenum atom, with a stoichiometry of one MPT per Mo. Moco is present

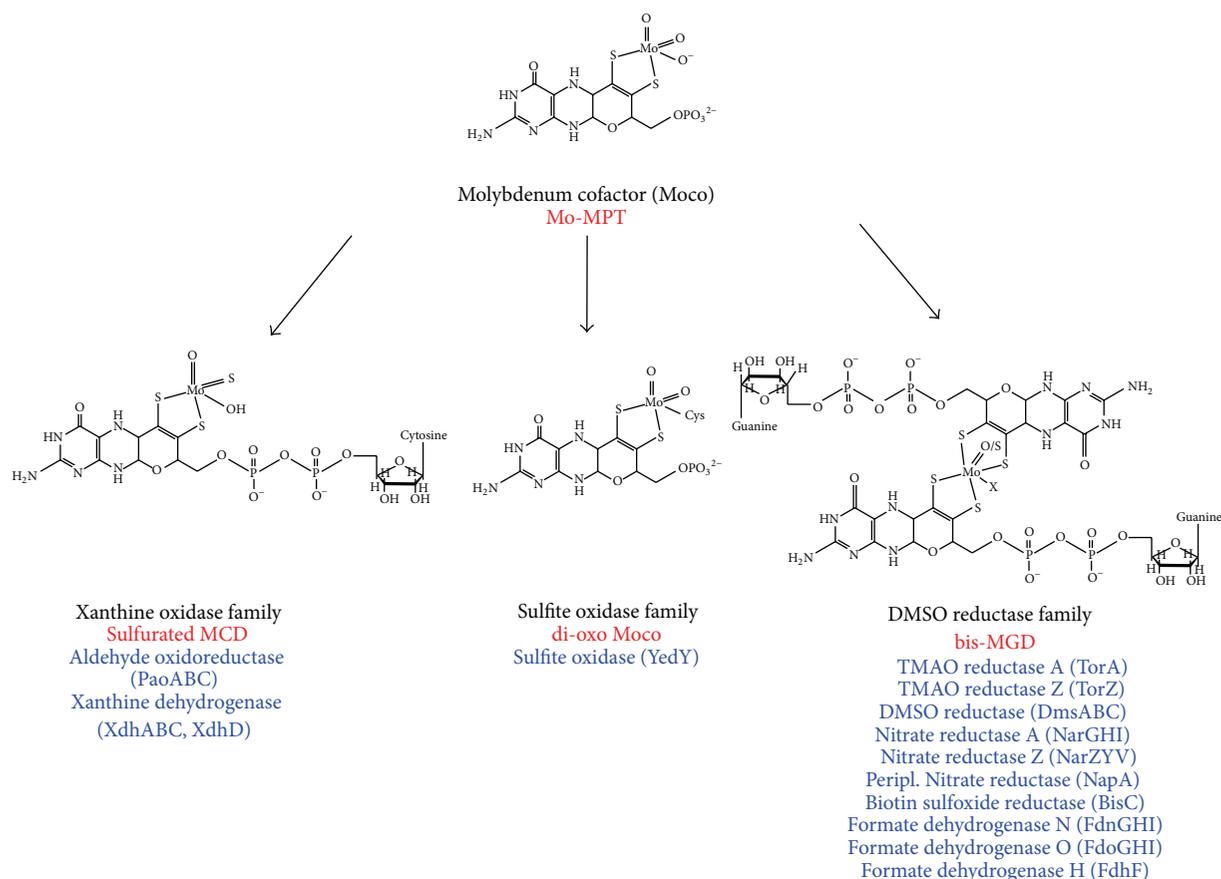


FIGURE 1: The different structures of Moco in *E. coli*. The basic form of Moco is a 5,6,7,8-tetrahydropyranopterin with a unique dithiolene group coordinating the molybdenum atom, named Mo-MPT. Mo-MPT (shown in the tri-oxo structure [25]) can be further modified and in *E. coli* three different molybdenum-containing enzyme families exist classified according to their coordination at the molybdenum atom: the xanthine oxidase, sulfite oxidase, and DMSO reductase families. In *E. coli*, the xanthine oxidase family contains the sulfurated MCD cofactor. 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. The characterized molybdoenzymes in *E. coli* are shown in blue for each family.

in more than fifty enzymes in bacteria, including nitrate reductases, sulfite dehydrogenase, xanthine oxidoreductase, aldehyde oxidoreductase, DMSO reductase, formate dehydrogenase, CO dehydrogenase, and various other enzymes [3]. In the redox reactions catalyzed by molybdoenzymes electron transfer is mediated by additional redox centers present in the protein or on other protein domains, like FeS centers, cytochromes, or FAD/FMN cofactors [6]. During these redox reactions, the molybdenum atom can exist in various oxidation states and couple oxide or proton transfer with electron transfer [2].

The different enzymes contain different forms of Moco and were historically categorized into three families based on the ligands at the molybdenum atom, which are characteristic for each family (Figure 1): the xanthine oxidase (XO) family, the sulfite oxidase (SO) family, and the dimethyl sulfoxide (DMSO) reductase family [3, 7]. The XO family is characterized by an MPT-Mo<sup>VI</sup>OS(OH) core in the oxidized state,

with one MPT equivalent coordinated to the molybdenum atom, one oxo-group, one sulfido-group, and one hydroxyl-group [3] (Figure 1). The sulfido-group is cyanide labile [8–10], and 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 [10]. The enzymes of this family are involved in two-electron transfer hydroxylation and oxo-transfer reactions with water as the source of oxygen [7, 11]. Among the members of the XO family in *E. coli* are the xanthine dehydrogenase XdhABC, the periplasmic aldehyde oxidoreductase PaoABC, and the so far uncharacterized xanthine dehydrogenase homologue XdhD (Figure 1, Table 1) [6, 12].

Enzymes of the SO family coordinate a single equivalent of the pterin cofactor with an MPT-Mo<sup>VI</sup>O<sub>2</sub> core in its oxidized state and usually an additional cysteine ligand which is provided by the polypeptide [3]. Members of this family catalyze the transfer of an oxygen atom either to or from

TABLE 1: Overview on the *E. coli* molybdoenzymes and the involved chaperones required for maturation/cofactor insertion.

Mo-enzyme	Subunits	Chaperone	Moco
<i>DMSO reductase family</i>			
Nitrate reductase A	NarGHI	NarJ	bis-MGD
Nitrate reductase Z	NarZYV	NarW	bis-MGD
Peripl. nitrate reductase	NapABCGH	NapD	bis-MGD
TMAO reductase A	TorAC	TorD	bis-MGD
TMAO reductase Z	TorZY	?	bis-MGD
DMSO reductase	DmsABC	DmsD	bis-MGD
Formate dehydrogenase N	FdnGHI	FdhD	bis-MGD
Formate dehydrogenase O	FdoGHI	FdhD	bis-MGD
Formate dehydrogenase H	FdhF	FdhD	bis-MGD
Biotin sulfoxide reductase	BisC	—	bis-MGD
Selenate reductase	YnfEFGH	DmsD	bis-MGD
?	YdhYVWXUT	?	?
?	YdeP	?	?
<i>Xanthine oxidase family</i>			
Aldehyde oxidoreductase	PaoABC	PaoD	MCD
Xanthine dehydrogenase	XdhABC	YqeB	MCD
?	XdhD (YgfM)	YqeB	?
<i>Sulfite oxidase family</i>			
Sulfite oxidase	YedYZ	—	Mo-MPT
?	YcbX	—	?
?	YiiM	—	?

—: no chaperone identified.

?: function unknown or not clear which chaperone is involved in maturation, form of Moco not characterized.

the substrate [13]. Among the members of this family in *E. coli* is the YedY protein (Figure 1 and Table 1), for which the physiological role is unknown so far [14].

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 [3]. The molybdenum coordination sphere is usually completed by a single Mo=O group with a sixth ligand in the  $\text{MPT}_2\text{-Mo}^{\text{VI}}\text{O(X)}$  core; however, a sulfido ligand has been recently described to replace the oxo-group in formate dehydrogenase-H from *E. coli* [15]. The sixth ligand, X, can be a serine, a cysteine, a selenocysteine, an aspartate or a hydroxide, and/or water molecule [16]. 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* (Figure 1 and Table 1).

This review will give in the first part an overview on the recent discoveries in Moco biosynthesis in *E. coli*. Since MPT is a sulfur-containing cofactor, the biosynthesis of Moco is linked to the sulfur mobilization in the cell. Two sulfurs are present in MPT which coordinate the molybdenum

atom. This sulfur is mobilized from L-cysteine in the cell, by a general pathway which links Moco biosynthesis to the synthesis of other sulfur-containing biomolecules like FeS cluster assembly and the thiolation of tRNA. Additionally, FeS cluster containing proteins are required for the biosynthesis of the first intermediate in Moco biosynthesis. Thus, we will dissect the sulfur transfer pathway for the biosynthesis of Moco and FeS clusters and their connection to the thiolation of tRNA in the second part of the review. The biosynthesis of the molybdenum cofactor in eukaryotes has been additionally studied and is also highly conserved. Since there are numerous reviews available in the literature describing Moco biosynthesis in humans and plants [17–20], we focus only on the biosynthesis in bacteria and the newly discovered connection to other biosynthetic pathways.

## 2. The Biosynthesis of the Molybdenum Cofactor in *E. coli*

Using a combination of biochemical, genetic, and structural approaches, Moco biosynthesis has been extensively studied for decades. In all bacteria Moco is synthesized by a conserved biosynthetic pathway that can be divided into four steps, according to the stable biosynthetic intermediates which can be isolated and were intensively studied in *Escherichia coli* (Figure 2) [21]: the synthesis of cyclic pyranopterin monophosphate (cPMP) [22], conversion of cPMP into MPT by introduction of two sulfur atoms [23], and insertion of molybdate to form Moco [24]. In bacteria, Moco is further modified by the attachment of GMP or CMP to the phosphate group of MPT, forming the dinucleotide variants of Moco, MPT-guanine dinucleotide (MGD) [25–27], and MPT-cytosine dinucleotide (MCD) [28]. After Moco biosynthesis, the different forms of Moco are inserted into the specific target enzymes by the help of molecular Moco-binding chaperones.

In *E. coli* five gene loci were identified which are directly involved in the biosynthesis of Moco, *moa*, *mob*, *moc*, *moe*, and *mog*, comprising 11 genes in total [21, 29] (Figure 3). The *moa* locus contains the genes *moaABCDE*, whose gene products are involved in cPMP formation from 5'GTP and in the formation of MPT [30]. The *mob* locus contains the genes *mobAB*, whose gene products are involved in the synthesis of bis-Mo-MPT and bis-MGD from Mo-MPT [31]. The *moc* locus contains the gene *moaA*, and the MoeA protein is involved in MCD synthesis from Mo-MPT [28]. The *moe* locus contains the *moeA* and *moeB* genes [32]. While MoeA is involved in Mo-MPT formation from MPT-AMP [33], MoeB is involved in the activation of MoeD by adenylation [34]. The *mog* locus contains the *mogA* gene, whose gene product is involved in the formation of MPT-AMP [33, 35]. Additional essential reactions are the transport of molybdate [36] and the mobilization of sulfur [37]. The proteins and biochemical reactions involved in the four steps of Moco biosynthesis are described in detail below.

**2.1. Conversion of 5'GTP to cPMP.** The biosynthesis of Moco starts from 5'-GTP, which results in the formation of cPMP,

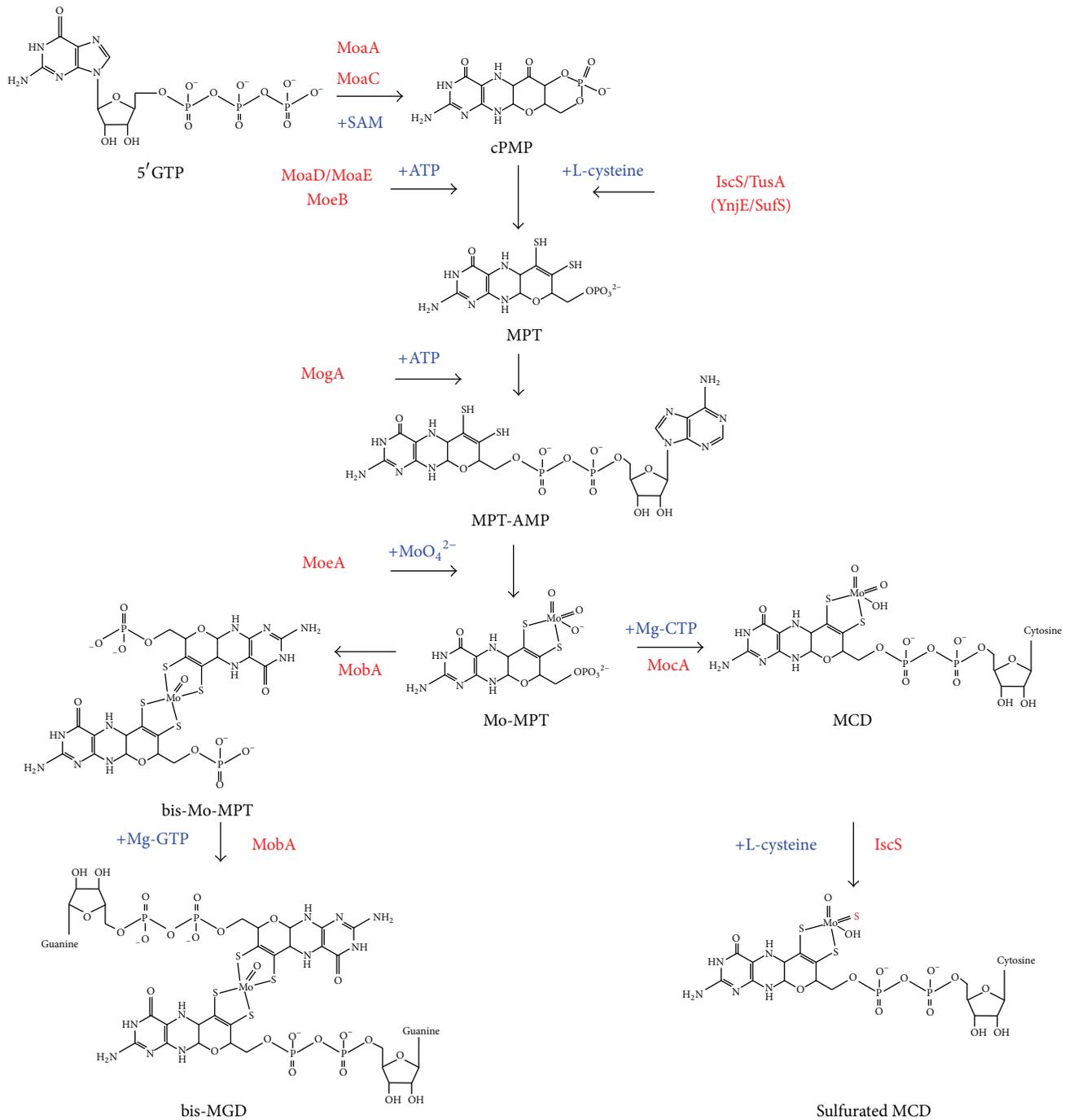


FIGURE 2: The biosynthesis of Moco in *E. coli*. Shown is a scheme of the biosynthetic pathway for Moco biosynthesis in *E. coli* and the proteins involved in this pathway. Mo-MPT is formed from 5' GTP with cPMP, MPT, and MPT-AMP as intermediates. Mo-MPT is directly inserted into enzymes of the sulfite oxidase family. For enzymes of the DMSO reductase family, Moco is further modified by formation of a bis-Mo-MPT intermediate and further addition of a GMP molecule to each MPT unit, forming the bis-MGD cofactor. Both reactions are catalyzed by the MobA protein. For enzymes of the xanthine oxidase family in *E. coli*, Mo-MPT is further modified by the addition of CMP to form the MCD form of the cofactor. 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, and additional molecules required for the reactions are shown in blue.

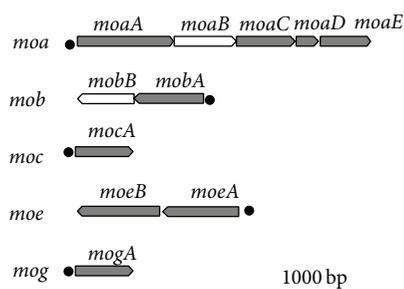


FIGURE 3: Organization of the genes involved in the biosynthesis of Moco in *E. coli*. In total, 11 genes are involved in the biosynthesis of Moco in *E. coli*. These are organized into 5 different gene loci termed *moa*, *mob*, *moc*, *moe*, and *mog*. Genes of known function are colored in white. Black dots indicate promoter regions. The genes are drawn approximately to scale. Additional operons with a role for in Moco biosynthesis, such as the *isc* operon or the *mod* operon for molybdate transport, are not shown.

the first stable intermediate of Moco biosynthesis (Figure 4) [22]. The cPMP molecule is an oxygen-sensitive 6-alkyl pterin with a cyclic phosphate group at the C2' and C4' atoms [38, 39]. In *E. coli*, the *moaA* and *moaC* gene products are responsible for the complicated chemical reactions required to generate cPMP [32, 40–42]. An *in vitro* system for cPMP synthesis containing the MoaA and MoaC proteins purified from *Staphylococcus aureus* showed that 5'-GTP is the specific initial substrate for cPMP biosynthesis [43]. In this reaction, the C8 of GTP is inserted between the C2' and C3' carbons of the GTP ribose. MoaA belongs to the superfamily of S-adenosyl methionine- (SAM-) dependent radical enzymes [44]. Members of this family catalyze the formation of protein and/or substrate radicals by reductive cleavage of SAM by [4Fe4S] cluster [45]. MoaA is a protein containing two oxygen-sensitive FeS clusters, each of which is coordinated by only three cysteine residues. The N-terminal [4Fe4S] cluster, present in all radical SAM proteins, binds SAM and carries out the reductive cleavage of SAM to generate the 5'-deoxyadenosyl radical, which subsequently initiates the transformation of 5'-GTP bound through the C-terminal [4Fe4S] cluster [44, 46, 47]. Experiments with GTP isotopes showed that the ribose C3' hydrogen atom is abstracted by the 5'-deoxyadenosyl radical of MoaA [48]. Further reactions involve the attack of the C8 in the guanine ring by the formed C3' radical resulting in the formation of (8S)-3', 8-cyclo-7,8-dihydroguanosine 5'triphosphate (3',8-cH<sub>2</sub>GTP) intermediate (Figure 4) [40, 41]. The additional reducing equivalents required for this step might be provided by the C-terminal [4Fe4S] cluster in MoaA. The intermediate serves as a substrate for MoaC which converts 3',8-cH<sub>2</sub>GTP to cPMP, including pyrophosphate cleavage and formation of the cyclic phosphate group by general acid/base catalysis [40].

**2.2. Conversion of cPMP to MPT.** The next step involves the conversion of cPMP to MPT in which two sulfur atoms are incorporated in the C1' and C2' positions of cPMP [23, 38, 49] (Figure 5). This reaction is catalyzed by MPT synthase, a protein consisting of two small (~10 kDa) and two large

subunits (~21 kDa), encoded by *moaD* and *moaE*, respectively [50, 51]. It was shown that MPT synthase carries the sulfur in form of a thiocarboxylate at the C-terminal glycine of MoaD [52]. The central dimer is formed by two MoaE subunits containing one MoaD at each end, as revealed by the crystal structure [53]. It was shown that the two MoaD/MoaE dimers act independently. Thus, for the insertion of two sulfurs into cPMP, two MoaD proteins are required at each end of the MPT synthase tetramer [54]. The first sulfur is added by one MoaD molecule at the C2' position of cPMP (Figure 5), a reaction which is coupled to the hydrolysis of the cPMP cyclic phosphate [55]. During the course of this reaction, a hemisulfurated intermediate is formed in which the MoaD C-terminus is covalently linked to the substrate via a thioester linkage, which subsequently is hydrolyzed by a water molecule. After the transfer of its thiocarboxylate sulfur to cPMP, the first MoaD subunit dissociates from the MPT synthase complex [54, 55]. During 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 the attack by the second MoaD thiocarboxylate (Figure 4). 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. During the reaction, cPMP and the hemisulfurated intermediate remain bound to one MoaE subunit [56].

The regeneration of sulfur at the C-terminal glycine of MoaD is catalyzed by MoeB [32, 57] and resembles the first step of the ubiquitin-dependent protein degradation system [58] (Figure 4). 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 [37]. After the reaction, MoaD-SH dissociates from the complex and reassociates with MoaE to form active MPT synthase (Figure 5). The binding constants within the different complexes of MoaD were shown to follow the order (MoaD-SH-MoaE)<sub>2</sub> > (MoaD-MoeB)<sub>2</sub> > (MoaD-MoaE)<sub>2</sub> [56, 59]. 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)<sub>2</sub> complex is stabilized by ATP addition and the subsequent formation of the acyl-adenylate on MoaD [34]. In this form, the sulfur transfer to MoaD occurs, generating MoaD-SH. In the sulfur transfer reactions the proteins IscS and TusA are involved, forming a sulfur relay system [60]. However, under anaerobic conditions, TusA can be replaced by YnjE and SufS [61]. The exact mechanism of sulfur mobilization and transfer is further described below. After the formation of the (MoaD-SH-MoaE)<sub>2</sub> 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 (Figure 2).

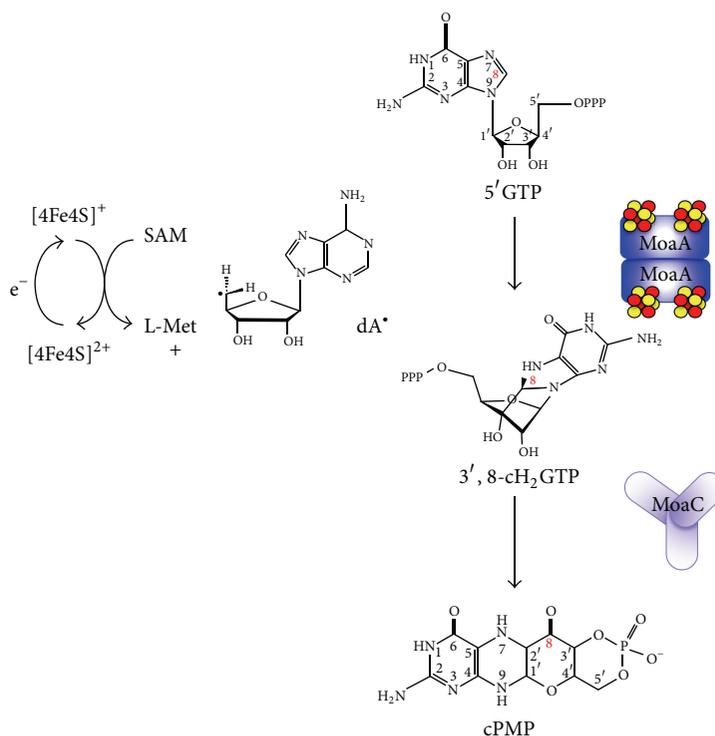


FIGURE 4: Synthesis of cPMP from 5' GTP. All carbon atoms of the 5' GTP are found within cPMP. The C8 atom from the guanine ring is inserted between the C2' and C3' atoms of the ribose. This reaction is catalyzed by the MoaA protein, an S-adenosylmethionine- (SAM-) dependent enzyme. MoaA forms a dimer with two [4Fe4S] clusters bound to each monomer. The trimeric MoaC protein is suggested to cleave the pyrophosphate group of the cyclic intermediate. cPMP is shown in the tetrahydropyrano form with a keto group at the C1' position, as suggested from the crystal structure [55].

**2.3. Insertion of Molybdate into MPT.** In *E. coli* insertion of the metal into MPT is accomplished by the *moeA* and *mogA* gene products (Figure 2) [24, 32]. The structure showed that MogA is a trimer in solution with each monomer folded into a single compact domain, and MogA binds MPT with high affinity [62]. 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 [63, 64]. It was shown that the proteins have different functions in the molybdenum chelation reaction [33, 65]. MoeA appeared to mediate molybdenum ligation to newly synthesized MPT *in vitro* at low concentrations of  $\text{MoO}_4^{2-}$ . 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 [65]. Later, the catalytic formation of an MPT-AMP intermediate during the reaction was shown by the crystal structure of the homologous protein Cnx1 from *Arabidopsis thaliana* with bound MPT-AMP [35, 66, 67]. The accumulation of a comparable MPT-AMP intermediate in *E. coli moeA*<sup>-</sup> extracts was verified later (Figure 2, unpublished data). After *in vitro* incubation of MoeA with Mo-MPT, EXAFS studies showed that Mo-MPT is bound in its tri-oxo structure to MoeA [25].

It was shown that under physiological molybdate concentrations (1–10  $\mu\text{M}$ ), MogA is required in *E. coli* to form an MPT-AMP intermediate that facilitates molybdate insertion on the dithiolene sulfurs. However, this reaction is not

absolutely required under high molybdenum concentrations *in vivo*, since *mogA*<sup>-</sup> cells were rescued for molybdoenzyme activities by the addition of high molybdate concentrations to the medium [68]. This suggested that, under high molybdate concentrations (>1 mM) in the cell, MPT-AMP formation by MogA is not required and molybdate can be directly inserted into MPT with the aid of the MoeA protein [69]. Additionally, it was shown that bivalent copper and cadmium ions as well as trivalent arsenite ions could all be inserted 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 [69]. Thus, bivalent metal ions in high concentrations might inhibit Moco biosynthesis in *E. coli*. After its formation, the Mo-MPT cofactor can be directly inserted into enzymes from the sulfite oxidase family without further modification [68], like into the *E. coli* YedY protein (Figure 1) [70].

#### 2.4. Further Modification of Moco

**2.4.1. bis-Mo-MPT and bis-MGD Formation for Enzymes of the DMSO Reductase Family.** The proteins of the DMSO reductase family in *E. coli* contain a dinucleotide derivative of Moco, the MPT-guanine dinucleotide (MGD) cofactor [3]. Additionally, the molybdenum atom is ligated by two dithiolene groups of two MGD moieties, forming the bis-MGD cofactor [27]. The synthesis of the bis-MGD cofactor

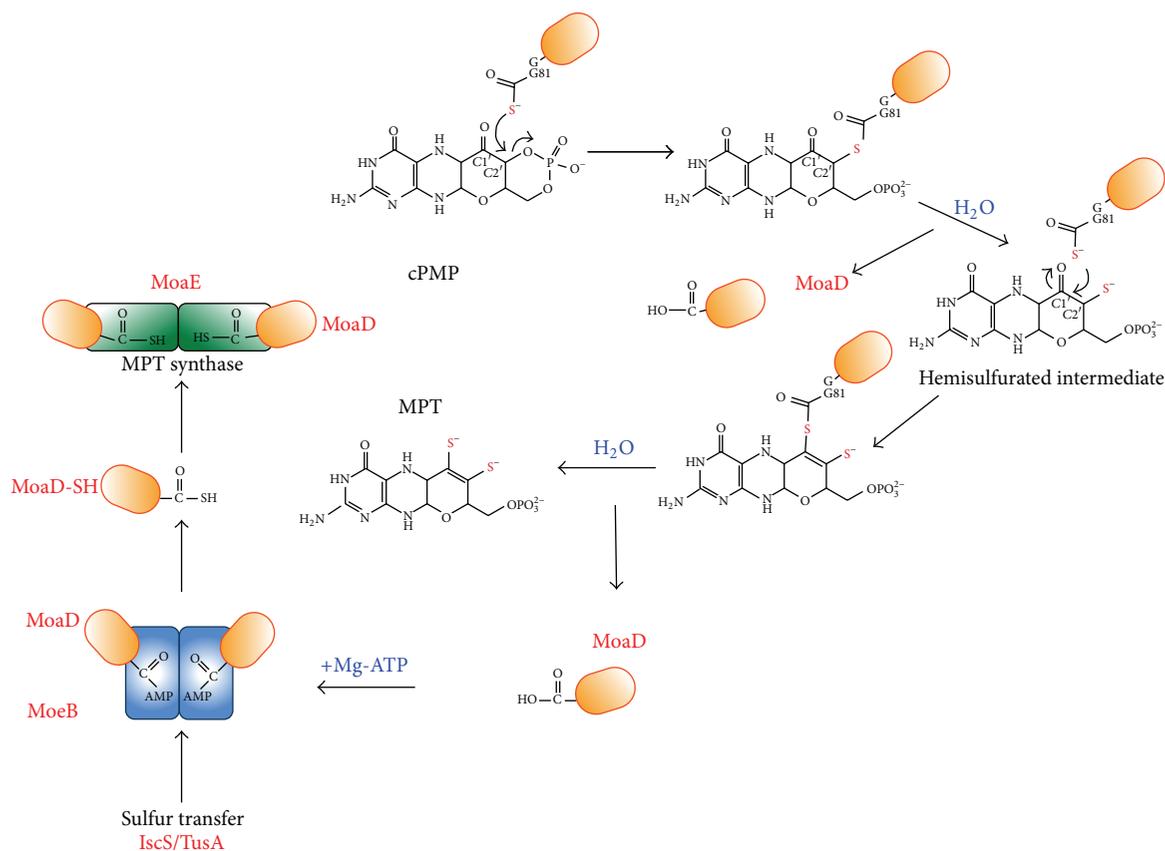


FIGURE 5: The biosynthesis of MPT from cPMP. In the MPT synthase mechanism, cPMP is bound to the MoaE subunit. The initial attack and transfer of the first thiocarboxylated MoaD-SH sulfur atom occurs at the C2' position of cPMP, coupled to the hydrolysis of the cPMP cyclic phosphate. An intermediate 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 to generate a hemisulfurated intermediate at C2'. Opening of the cyclic phosphate shifts the location of the intermediate within the complex to a position where C1' becomes more accessible. A new MoaD-SH thiocarboxylate attacks the C1' resulting in a second covalent intermediate which is converted to MPT via the elimination of a water molecule and hydrolysis of the thioester intermediate. During the reaction, cPMP remains bound to the MoaE molecule. The MPT synthase tetramer is built of two MoaE and two MoaD subunits. The MoaD-SH thiocarboxylate is formed on MoeB, where MoaD is first activated under ATP consumption to form an activated MoaD acyl-adenylate. Further, sulfur is transferred from a sulfur relay system by an L-cysteine desulfurase in conjunction with either TusA or YnjE to MoaD. The mechanism was adapted from the one proposed in [55].

occurs in a stepwise reaction which only requires Mo-MPT (which was previously formed by the MogA/MoeA reaction), MobA, and Mg-GTP [25]. In the first reaction, the bis-Mo-MPT intermediate is formed on MobA (Figure 2). In the second reaction, two GMP moieties from GTP are added to the C4' phosphate of each MPT via a pyrophosphate bond leading to release of the  $\beta$ - and  $\gamma$ -phosphates of GTP as pyrophosphate [71]. However, the molecular mechanism of bis-Mo-MPT formation as well as its binding mode to MobA is not clear so far. The crystal structure of MobA showed that the protein is a monomer with an overall  $\alpha\beta$  architecture, in which the N-terminal domain of the molecule adopts a Rossmann fold, and a possible MPT binding site that is localized to the C-terminal half of the protein [72, 73]. Since for bis-Mo-MPT formation two MPT moieties have to be bound to monomeric MobA, this might occur by using the MPT and predicted GTP-binding sites (Figure 6). During

this reaction, one molecule of molybdate has to be released when two Mo-MPT molecules are combined; however, the underlying chemistry remains elusive and the release of molybdate has not been proven so far. It also remains possible that MobA binds one Mo-MPT molecule and one MPT molecule from which the bis-Mo-MPT could be formed. After the attachment of two GMP molecules to the bis-Mo-MPT intermediate, bis-MGD is formed [25]. Facilitated release of bis-MGD from MobA in the presence of GTP was observed, which suggested that GTP might compete with the same binding site occupied by the MPT units, thus, resulting in the release of formed bis-MGD. We propose that two MPT moieties bind to both the predicted GTP-binding sites in addition to the predicted MPT binding site on MobA, thus, enabling bis-Mo-MPT and bis-MGD synthesis by monomeric MobA (Figure 5). The favourable release only of the final product may then be induced by a different binding mode of

bis-MGD compared to bis-Mo-MPT to MobA. Both binding modes can be modeled on MobA, which is shown in Figure 6.

While the role of MobA in MGD formation of Moco was already discovered in 1991 [74], the role of MobB, the second protein encoded by the *mob* locus [31], remains uncertain [75]. 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 [75]. Another possible role might be that MobB protects formed bis-MGD or prevents its release from MobA until an acceptor protein is present. A docking model of MobA and MobB suggested that GTP is bound to a shared binding site at the interface between both proteins [75, 76]. However, conditions under which MobB is essential for bis-MGD synthesis were not reported so far. However, in some bacteria like *Rhodobacter capsulatus*, MobB is not present and, thus, is not essential for bis-MGD formation [77]. After bis-MGD biosynthesis, the cofactor is released from MobA and either is bound to Moco-binding chaperones or is inserted into the target molybdoenzymes of the DMSO reductase family [71, 78].

**2.4.2. Molecular Chaperones for bis-MGD Insertion into Target Enzymes.** The last step of Moco modification including the formation of bis-MGD prepares the cofactor for insertion into the specific apo-enzymes. Until now it is not completely understood how Moco is inserted into the folded protein. The crystal structures of several molybdoenzymes revealed that Moco is deeply buried inside the proteins, at the end of a funnel-shaped passage giving access only to the substrate [79]. The insertion step is catalyzed by Moco-binding molecular chaperones, which bind the respective Moco variant and insert it into the target molybdoenzyme [78]. With a few exceptions, most of the molybdoenzymes of the DMSO reductase family in *E. coli* have a specific chaperone for Moco insertion (Table 1) [6]: NarJ is the chaperone for nitrate reductase A NarGHI [80], NarW is the chaperone for nitrate reductase Z NarZYV [81], DmsD is the chaperone for DmsABC [82] and YnfE/F [83], and FdhD is the chaperone for FdhF [84]. One well-studied example is the TorD/TorA system for TMAO reductase in *E. coli*. TorD was shown to be the specific chaperone for TorA [85] and plays a direct role in the insertion of Moco into apoTorA [86]. During this reaction, TorD interacts with both MobA and apoTorA and further stabilizes apoTorA for Moco insertion to avoid a proteolytic attack of the latter. This is consistent with its role as “facilitator” of the bis-MGD insertion and maturation of the apo-enzyme [6, 78, 87]. For the chaperone TorD it was described that it is able to bind to the signal peptide of apo-TMAO reductase until the bis-MGD is inserted and TMAO reductase is correctly folded [87]. Pre-TorA is then translocated to the periplasm where the active TorA enzyme is finally generated after cleavage of the signal peptide [6].

Recently, it was shown that these chaperones not only facilitate the insertion of bis-MGD into the target enzyme, but also directly bind bis-MGD [88]. This was shown by the FdsC-FdsA system for maturation of *R. capsulatus* formate

dehydrogenase (FDH). *R. capsulatus* FDH consists of  $(\alpha\beta\gamma)_2$  heterotrimer in which the large  $\alpha$ -subunit FdsA (105 kDa) harbors the bis-MGD cofactor and a set of four  $[\text{Fe}_4\text{S}_4]$  clusters and one  $[\text{Fe}_2\text{S}_2]$  cluster (Figure 7). FdsA is linked to the  $\beta$ -subunit FdsB (52 kDa) that binds one additional  $[\text{Fe}_4\text{S}_4]$  cluster and the FMN cofactor [89]. The  $\gamma$ -subunit FdsG (15 kDa) binds  $[\text{Fe}_2\text{S}_2]$  cluster. The terminal electron acceptor was shown to be  $\text{NAD}^+$ . For *R. capsulatus* FDH so far two proteins, named FdsC and FdsD, were identified to be essential for the production of an active FDH, but are not subunits of the mature enzyme [89]. While FdsD has only counterparts in some oxygen-tolerant FDHs, FdsC shares high amino acid sequence homologies to *E. coli* FdhD, the chaperone for the membrane-bound FDH (FdhF) [84]. FdsC was directly copurified with bound bis-MGD [88]. The definitive proof that bis-MGD was bound to FdsC and not only MGD was given by the reconstitution of *E. coli* TMAO reductase activity in a system solely consisting of FdsC and apo-TMAO reductase. It also was concluded that, in addition to bis-MGD binding, FdsC might have a similar role in the maturation of FDH like TorD for TMAO reductase. For TorD it was shown that it interacts with MobA and TorA [78, 87]. For FdsC an interaction with MobA, FdsD, and FdsA was shown. Thus, FdsC might also act as a platform connecting bis-MGD biosynthesis and its insertion into the target protein (Figure 7). FdsC binds directly bis-MGD and therefore might protect the bis-MGD cofactor from oxidation before its insertion into FdsA. Alternatively, it might be the factor that determines the specificity for bis-MGD insertion into FDH. The FdsD protein seems to be additionally essential in this reaction. Since FdsD was only identified in organisms which contain an oxygen-tolerant FDH, its role might also serve to protect the bis-MGD cofactor specifically in the presence of oxygen.

Additionally, it was reported that the bis-MGD cofactor can be further modified by sulfuration [15, 16, 90, 91]. 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 [15]. In the oxidized state, the enzyme contains the four pterin sulfur ligands at the Mo site, a selenocysteine ligand, and a  $-\text{SH}$  ligand. The chaperone involved in sulfuration of the Moco for FdhF was shown to be the FdhD protein [84]. For the *E. coli* FdhD protein it was reported that it acts as a sulfurtransferase between the L-cysteine desulfurase IscS and FdhF, a mechanism which is essential to yield active FdhF [84]. Conclusively, the additional role of bis-MGD binding chaperones might be the further modification of Moco by sulfuration. 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 [90]. 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 [91]. These observations might suggest that sulfuration

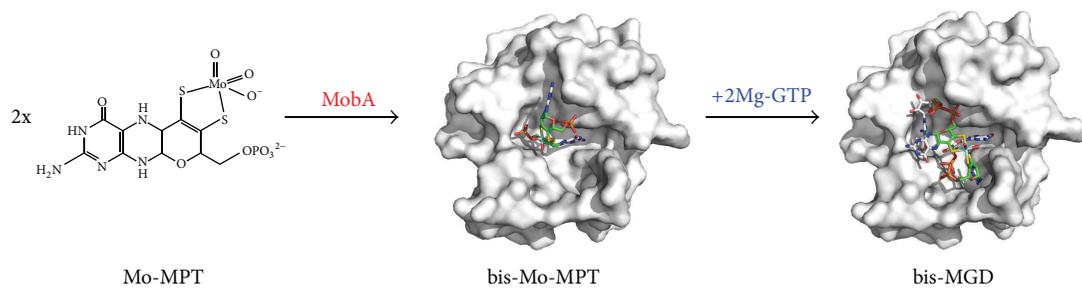


FIGURE 6: Model of the formation of bis-Mo-MPT and bis-MGD by MobA. It is proposed that two Mo-MPT moieties bind to the proposed MPT and GTP-binding sites on the MobA monomer, thus, enabling first bis-Mo-MPT and then bis-MGD synthesis by the addition of two GMP molecules to bis-Mo-MPT. bis-Mo-MPT and bis-MGD are believed to have different binding modes on MobA, resulting in the favourable release of bis-MGD as final product. The structures of MobA were modeled using the coordinates from the Protein Data Bank (1FRW) and the figure was adapted from the one shown in [25].

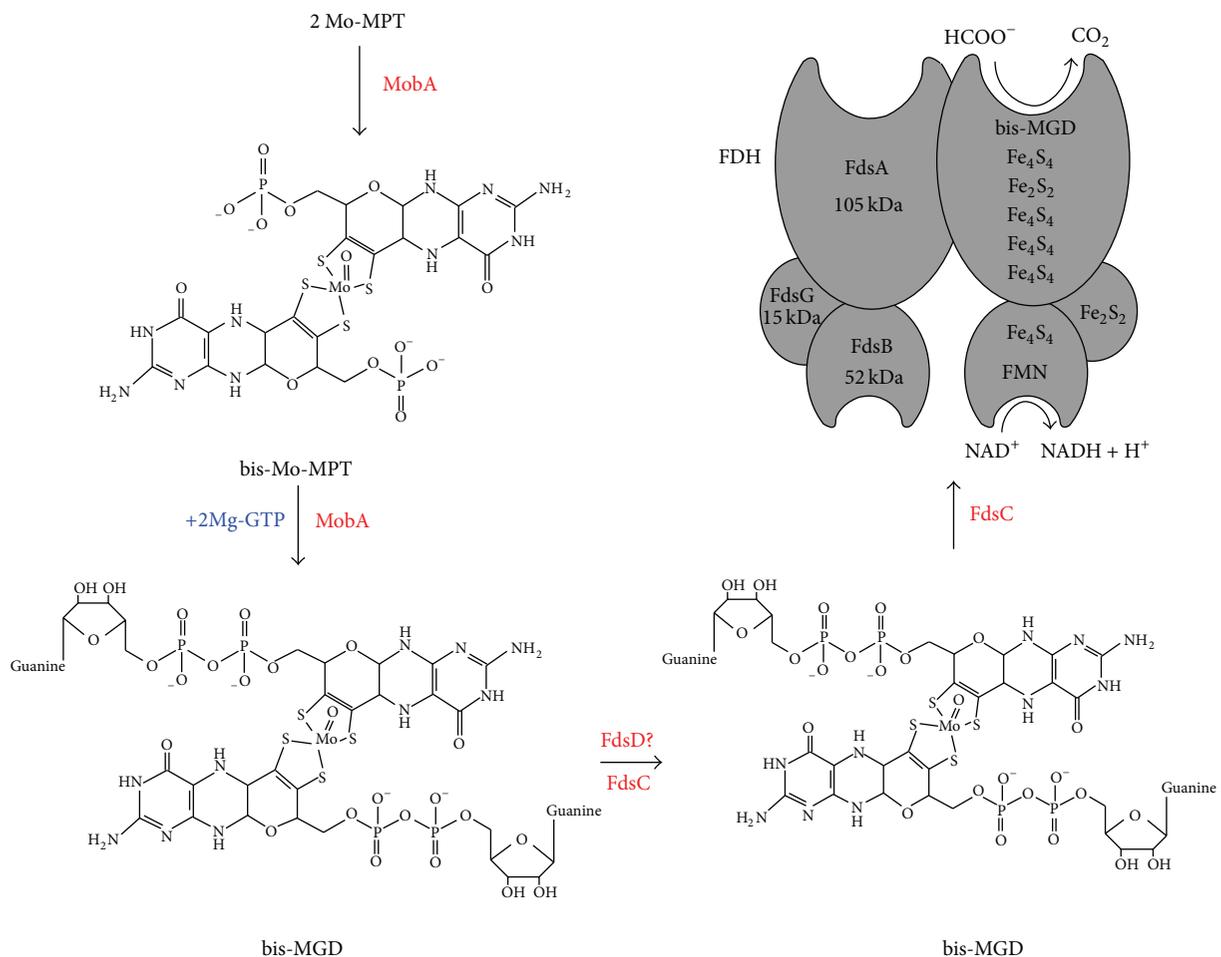


FIGURE 7: Model for the role of FdsC in bis-MGD insertion into *R. capsulatus* FDH. Shown is a scheme for bis-MGD biosynthesis from two Mo-MPT molecules via a bis-Mo-MPT intermediate. MobA binds Mo-MPT and catalyzes first the synthesis of bis-Mo-MPT and then the synthesis of bis-MGD by ligation of two GMP molecules to each MPT moiety in bis-Mo-MPT. Further, bis-MGD is transferred from MobA to FdsC. FdsC, FdsD, and MobA were shown to form a complex and are believed to protect the bis-MGD cofactor from oxidation before its insertion into the FdsA subunit of *R. capsulatus* FDH. Additionally, FdsC is believed to contribute to the specificity for bis-MGD insertion into FDH. The role of FdsD in the reaction is not completely resolved so far.

of bis-MGD is more common of this group of enzymes than previously expected. In total, we believe that bis-MGD binding to chaperones for enzymes of the DMSO reductase family might be a common feature, which might serve as a platform for further modification of Moco or the specific interaction with target enzymes for bis-MGD insertion.

**2.4.3. MCD Formation and Further Modification by MCD Sulfuration for Enzymes of the Xanthine Oxidase Family.** 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 (Figure 1) [92–94]. MCD formation is catalyzed by a protein which was named MocA, for molybdopterin cytosine dinucleotide synthesis (Figures 2 and 3) [28]. MocA was identified by amino acid sequence comparison to MobA, since they exhibit 22% amino acid sequence identity [28, 95]. The catalytic reaction of MocA is similar to the reaction of MobA, in that it acts as a CTP:molybdopterin cytidylyltransferase and covalently links MPT and CMP with the concomitant release of the  $\beta$ - and  $\gamma$ -phosphates of CTP as pyrophosphate [28]. However, bis-MCD is not the end product of the reaction; instead, only one MCD moiety is ligated to the molybdenum atom in this cofactor variant. Therefore, the end product of MocA must be a monopterin MCD cofactor. Instead, the MCD cofactor for enzymes of the xanthine oxidase family is further modified in *E. coli* and contains an equatorial sulfido ligand at its active site [12]. Thus, MCD sulfuration might prevent bis-MCD formation.

Comparison of the two MPT nucleotidyl transferases showed that MobA is highly specific for binding of the purine nucleotide GTP, while MocA is specific for binding of the pyrimidine nucleotide CTP [28]. 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 <sup>12</sup>LAGG<sup>15</sup> and <sup>78</sup>GPLAG<sup>82</sup> amino acid sequence segments [72, 96]. In MocA, these sequences are altered to <sup>12</sup>TAAG<sup>15</sup> and <sup>78</sup>GLLTS<sup>82</sup> [95]. Site directed mutagenesis studies showed 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 [95].

After MCD formation, the cofactor is handed over to MCD binding chaperones, which are PaoD or YqeB in *E. coli* (Table 1). PaoD is the specific Moco-binding chaperone for the periplasmic aldehyde oxidoreductase PaoABC in *E. coli* [6]. PaoD belongs to the XdhC family of Moco-binding chaperones [12]. The best characterized chaperone from this family is the *R. capsulatus* XdhC protein which is essential for the maturation of *R. capsulatus* xanthine dehydrogenase (XDH) [12]. Investigation of *R. capsulatus* XdhC showed that it binds the Moco produced by MoeA/MogA and protects

it from oxidation until the terminal molybdenum sulfur ligand is inserted [97, 98]. 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 [99]. The sulfur for this reaction originates from L-cysteine, and 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* ( $\alpha\beta$ )<sub>2</sub> XDH heterotetramer [98, 100]. Thus, it appears from the *R. capsulatus* studies that XdhC-like proteins perform a number of functions including stabilization of the newly formed Moco and interaction with an L-cysteine desulfurase to ensure that Moco sulfuration occurs [99] as well as interaction with their specific target proteins for insertion of the sulfurated Moco [12]. 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 [101]. This model implies that molybdoenzymes requiring the sulfurated form of Moco exist in a Moco competent “open” apo-molybdoenzyme conformation until the insertion of sulfurated Moco. After insertion, the protein adapts the final active “closed” conformation that can no longer accept Moco [101].

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 binds the MCD cofactor rather than an MPT cofactor (Figure 8) [102]. 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 (Table 1). 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 [12]. YqeB is a little larger in size than other members of the XdhC family and it contains an NAD(P) binding Rossmann fold. So far, no further data are available for YqeB.

### 3. The Distribution of Sulfur for Sulfur-Containing Biomolecules in Bacteria

L-Cysteine is the source for the biosynthesis of Moco and a variety of other biomolecules such as thiamin, iron-sulfur (FeS) clusters, and thionucleosides in transfer RNA (tRNA) (Figure 8) [103]. In *E. coli* the L-cysteine sulfur for these biomolecules is initially mobilized by the house-keeping L-cysteine desulfurase IscS [104]. The enzyme is a pyridoxal 5'-phosphate-containing homodimer that decomposes L-cysteine to L-alanine and sulfane sulfur via the formation of an enzyme-bound persulfide intermediate [104–106]. The persulfide sulfur from the L-cysteine desulfurase is further incorporated either directly or via sulfur relay systems into the biosynthetic pathways of several sulfur-containing biofactors, thus providing an elegant mechanism for making sulfur atoms available without releasing them in solution [107, 108]. IscS is encoded by a gene that is part of a larger

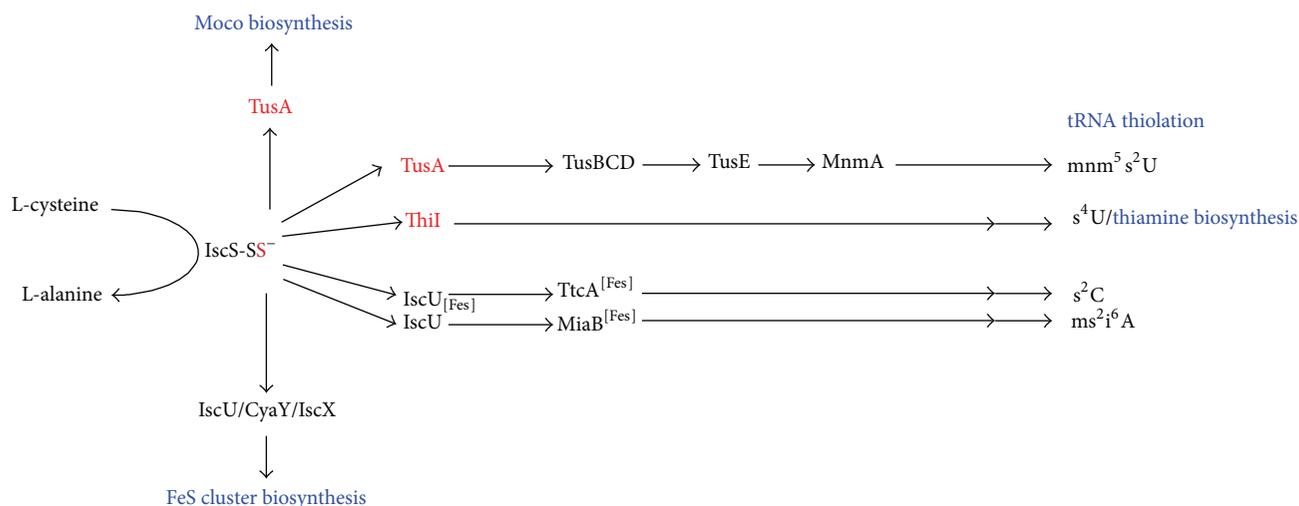


FIGURE 8: Sulfur transfer to sulfur-containing biomolecules involving the IscS-bound persulfide. A protein-bound persulfide group is formed on the L-cysteine desulfurase IscS, which is further transferred to proteins like IscU, TusA, or ThiI. CyaY and IscX were also shown to bind to IscS. IscU is the primary scaffold for the assembly of FeS clusters. The IscS sulfur can also be further transferred for the thiolation of tRNA. In *E. coli* tRNA modification can be divided into an FeS cluster independent pathway and into a pathway which requires FeS clusters. The synthesis of s<sup>2</sup>C by TtcA and ms<sup>2</sup>i<sup>6</sup>A by MiaB require sulfur from the FeS clusters initially formed on IscU. ThiI can transfer sulfur either for the formation of thiamine or for the formation of s<sup>4</sup>U in tRNA. TusA can transfer sulfur for a sulfur relay system involving TusBCD, TusE, and MnmA for the formation of s<sup>2</sup>U in tRNA, or additionally TusA is involved in sulfur transfer for Moco biosynthesis. Persulfide-containing proteins are highlighted in red and names of the final sulfur-containing molecules are colored in blue.

operon, *iscRSUA-hscBA-fdx-iscX*, in which the other gene products are involved in the biosynthesis of FeS clusters in *E. coli* (Figure 9) [104]. For FeS cluster biosynthesis IscS interacts with IscU, which serves as a scaffold protein for FeS cluster assembly [109, 110]. However, IscU is not the only interaction partner of IscS, since IscS was shown to interact with a number of other proteins for delivery of sulfur to other sulfur-containing molecules, such as CyaY, IscX, TusA, and ThiI (Figure 8) [107, 110]. While CyaY and IscX are also involved in FeS cluster biosynthesis [110–112], ThiI and TusA are involved in tRNA thiolation (ThiI and TusA) [113, 114], thiamine biosynthesis (ThiI) [115], or Moco biosynthesis (TusA) [60]. Thus, the persulfide sulfur from IscS can be transferred in a relay flow to the conserved cysteine residues of acceptor proteins [116]. This chapter will describe how the sulfur transfer pathways for FeS clusters, tRNA, and Moco are connected and which factors determine the direction of the sulfur transfer mechanism to the various biomolecules.

**3.1. The Biosynthesis of FeS Clusters.** FeS clusters are among the earliest catalysts in the evolution of biomolecules and serve as electron carriers in redox reactions, regulatory sensors, stabilizers of protein structure, and chemical catalysts [103]. The most abundant types are [2Fe2S] clusters and [4Fe4S] clusters. Although FeS clusters can be formed spontaneously *in vitro* with inorganic iron and sulfide [117], the *in vivo* synthesis is a complex process which is highly regulated in the cell [118].

The *isc* operon encodes for IscS, the scaffold protein IscU, ferredoxin (Fdx), the scaffold protein IscA, the two molecular chaperones HscA and HscB, and IscX a protein of unknown

function (Figure 9) [104]. The *isc* operon is controlled by IscR, FeS cluster binding transcriptional repressor [119]. The scaffold protein IscU is the central protein in FeS cluster biosynthesis and is responsible for the assembly of [2Fe2S] and [4Fe4S] clusters [109]. IscS provides the inorganic sulfur for FeS clusters [120]. In the presence of L-cysteine and Fe<sup>2+</sup>, IscS and IscU form a transient macromolecular complex and form sequentially [2Fe2S] clusters and then from two [2Fe2S] a [4Fe4S] cluster on IscU [121]. The sulfur is bound on IscS as a persulfide on Cys-328 and is subsequently transferred to the scaffold IscU. The crystal structure of the IscU-IscS complex showed that each IscU molecule interacts with one IscS molecule in a 1 : 1 stoichiometry [110]. In an intermediate structure, the Cys-328 residue of IscS is used as a ligand of the transient [2Fe2S] cluster together with the three cysteines on IscU [121]. Further, ferredoxin was proposed to participate in the reductive coupling of two [2Fe2S] clusters to form a single [4Fe4S] cluster on IscU [122]. The cluster is then subsequently transferred to a recipient apoprotein, which can be facilitated by the help of IscA [123]. The two chaperones HscA and HscB help in the formation and transfer of the [4Fe4S] cluster [124]. CyaY, the *E. coli* frataxin homologue, has also a role in FeS cluster biosynthesis and was shown to regulate the biosynthesis by competing about the binding site of IscS with other proteins like IscX [110, 112, 125]. Upon binding to IscS, CyaY was shown to inhibit FeS cluster biosynthesis. It has been suggested that CyaY binding may induce a conformational change in the IscS-IscU complex that does not significantly affect the IscS L-cysteine desulfurase activity, but abolishes FeS cluster synthesis [126]. This might occur by stabilizing a conformation on the flexible loop of IscS which disfavors FeS cluster assembly. The role of CyaY as an iron

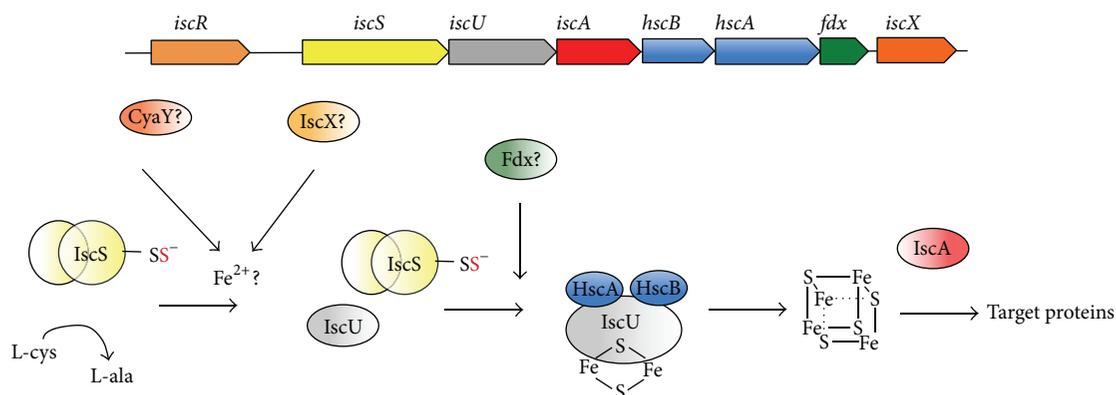


FIGURE 9: General scheme for FeS cluster biosynthesis in *E. coli*. Shown is the *isc* operon containing the genes *iscR-iscSUA-hscAB-fdx-iscX*. IscR is the regulator for expression of the operon by sensing the FeS status of the cell. FeS clusters assemble on the scaffold protein IscU, which receives the sulfur from the L-cysteine desulfurase IscS. The iron donor in this reaction is not identified so far. CyaY is believed to stabilize a conformation of the IscS-IscU complex. The role of IscX is unclear. Ferredoxin is proposed to participate in the reductive coupling of two [2Fe2S] clusters to form a single [4Fe4S] cluster on IscU. Release of the clusters is catalyzed by the chaperones HscAB. The carrier protein IscA delivers the formed FeS clusters to the final target proteins.

donor in this process is not completely resolved so far [127]. During stress conditions such as iron starvation and oxidative or heavy metal stress, the SufS protein is rather involved in biosynthesis of FeS clusters, replacing IscS in some functions [128, 129].

In total, FeS clusters are found to participate in diverse biological processes such as respiration, central metabolism DNA, or gene regulation. FeS clusters can act as catalysts or redox sensors and are predicted to be used by over 150 proteins in *E. coli* [118]. Among the FeS cluster containing molybdoenzymes are nitrate reductase, DMSO reductase and formate dehydrogenase, involved, for example, in anaerobic respiration [6].

**3.2. The Thiolation of tRNA.** TusA and ThiI are two proteins which are involved in the posttranscriptional modification of tRNA by producing thiolated nucleoside species at specific positions in the tRNA (Figure 8) [114, 130]. Thiolated nucleosides are found in several tRNAs. In *E. coli* these are  $s^4U8$ ,  $s^2C32$ ,  $ms^2i(o)^6A37$ , and  $mnm^5s^2U34$ , which, with the exception of  $s^4U8$ , are located within the anticodon loop and are crucial for proper mRNA decoding (Figure 10) [107]. The base thiolations are mediated by several acceptor proteins, which are divided into an FeS cluster dependent and an independent pathway. In the iron-sulfur cluster independent pathway, direct transfer of sulfur from IscS to the acceptor ThiI leads to the  $s^4U8$  modification at position 8 in bacterial tRNAs (Figure 10) [113, 131]. The  $s^4U$  serves as a near UV-photosensor since it undergoes photocrosslinking with a cytidine at position 13, which causes a growth delay since cross-linked tRNAs are inefficient aminoacylation substrates. However, ThiI has a dual role and also participates in thiamine biosynthesis by providing the sulfur of the thiazole species in thiamine pyrophosphate (Figure 12) [115].

In contrast, the TusA protein was identified to function as a sulfur mediator for the synthesis of 2-thiouridine of the modified wobble base 5-methylaminomethyl ( $mnm$ ) $s^2U$  in

tRNA (Figure 10) [114]. It interacts with IscS and stimulates its L-cysteine desulfurase activity 3-fold [114]. TusA transfers the sulfur from Cys19 to Cys78 of TusD in the TusBCD complex in a TusE-dependent manner. TusE is likely to accept the sulfur from TusD to form a persulfide of Cys108. Finally, TusE transfers the sulfur to MnmA, which directly incorporates sulfur into the tRNA wobble position. MnmA has a P-loop in its active site that recognizes tRNA and activates the C2 position of the uracil ring at position 34 by forming an adenylate intermediate [132–134]. In total, little is known about the function of the modification of 2-thiouridine. It is proposed that in *E. coli* thio-modified tRNA<sup>Lys</sup> confers efficient ribosome binding and 2-thio-modified tRNA<sup>Glu</sup> is required for specific recognition by glutamyl-tRNA synthetase. It also assists proper codon-anticodon base pairing at the ribosome A site and prevents frameshifting during translation. Thus, the 2-thio-modification of uridine 34 plays a critical role in the decoding mechanism [135].

The modification of tRNA also seems to affect lambda phage infection in *E. coli* [136, 137]. The process of phage development is dependent on tRNA thiolation. It was shown that a  $\Delta tusA$  mutant is more resistant to lambda phage infection because of a reduced tRNA modification efficiency and as a consequence has a defect in an associated frameshift reprogramming control. In contrast, *E. coli*  $\Delta hscA$ ,  $\Delta hscB$ , or  $\Delta iscU$  mutants were shown to be hypersensitive to lambda phage infection [137]. Inactivation of the FeS assembly machinery increases sulfur flux to TusA, resulting in an increased tRNA thiolation and as a consequence a “phage hypersensitivity” phenotype. Thus, tRNA modification and FeS cluster biosynthesis seem to be linked by the control of the sulfur flux to each pathway, which influence each other.

**3.3. Sulfur Transfer for Moco Biosynthesis.** The first step in Moco biosynthesis, the conversion of 5’GTP to cPMP, directly depends on the availability of FeS clusters [43]. The MoaA protein belongs to the class of radical SAM enzymes

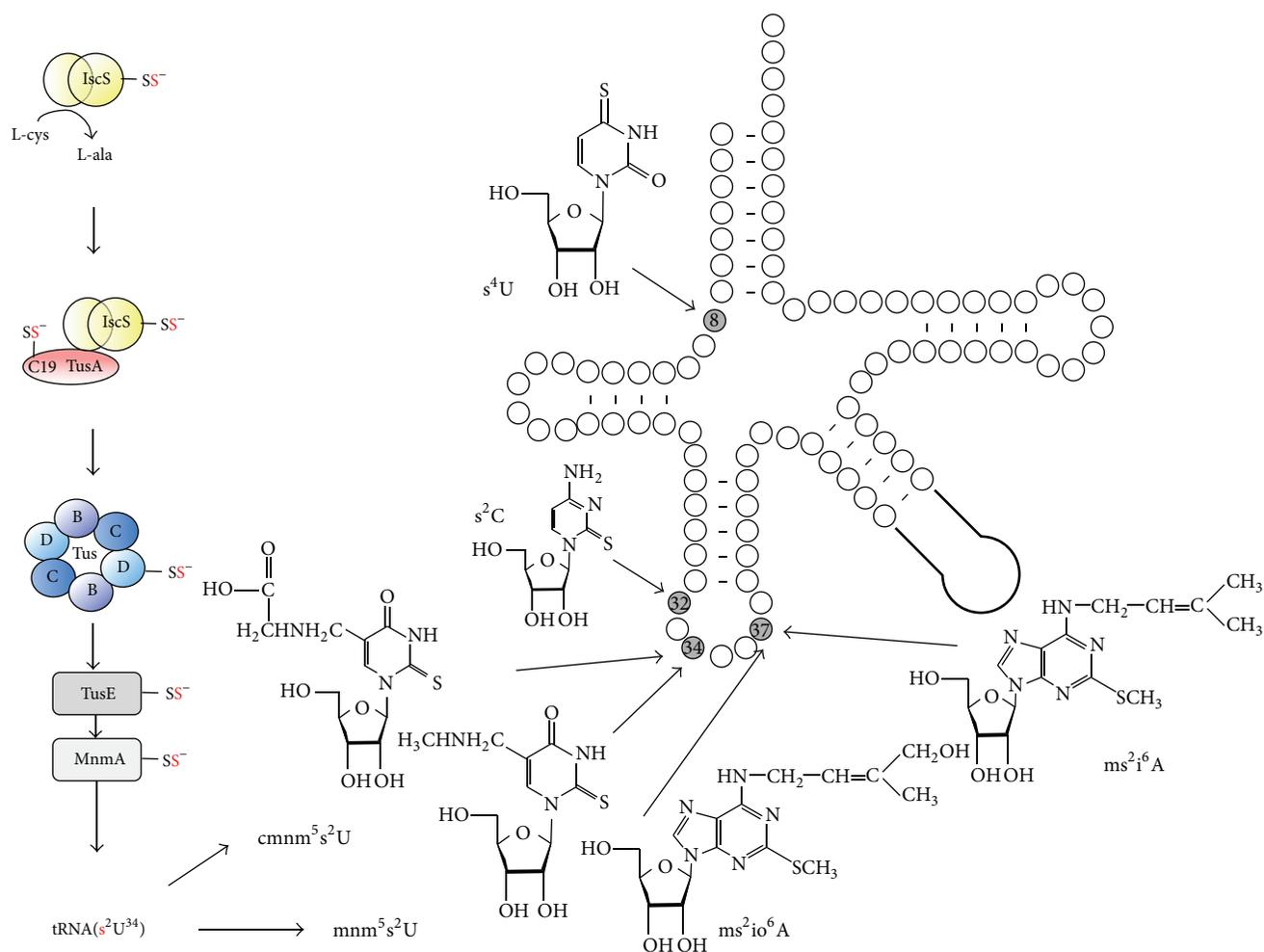


FIGURE 10: Chemical structure of thio-containing tRNA modifications. Shown are the secondary structure and the positions of thiolated nucleosides in tRNA. Abbreviations:  $s^4U$ , 4-thiouridine;  $s^2U$ , 2-thiocytidine;  $mmn^5s^2U$ , 5-methylaminomethyl-2-thiouridine;  $ms^2i^6A$ , 2-methylthio-N6-isopentenyladenosine;  $ms^2io^6A$ , N6-(4-hydroxyisopentenyl)-2-methylthioadenosine. Each number represents the nucleoside position in the tRNA. On the left-hand side the sulfur transfer pathway for the formation of  $s^2U$  is shown. The sulfur transfer involves the initial sulfur mobilization by IscS, sulfur transfer to TusA-C19, and further transfer via TusD in the TusBCD complex, TusE to MnmA, which binds and activates the tRNA by adenylation. After formation,  $s^2U$  is further modified to form  $cmnm^5s^2U$  and  $mmn^5s^2U$ .

and contains two [4Fe4S] clusters which are essential for its activity (Figure 4) [45]. Thus, when no FeS clusters are present in the cell, cPMP is not formed. Further, for the formation of MPT from cPMP, two sulfur molecules are inserted [53]. The primary sulfur for the dithiolene group of the MPT backbone of Moco was shown to be the IscS protein [37]. Further studies suggested that TusA is additionally involved in sulfur transfer for the synthesis of MPT, by a balanced regulation of the availability of IscS to various biomolecules in *E. coli* (Figure 5) [60]. It was shown that deletion of *tusA* in *E. coli* affected the activity of molybdoenzymes under aerobic and anaerobic conditions. Characterization of the  $\Delta tusA$  strain under aerobic conditions showed an overall low MPT content and an accumulation of cPMP. Under anaerobic conditions the activity of nitrate reductase was only 50% reduced, showing that TusA is not essential for Moco biosynthesis and can be replaced by other proteins. One sulfur-carrier is the rhodanese-like protein YnjE, which

was shown to be preferentially sulfurated by IscS [61]. The expression of *ynjE* is increased under anaerobic conditions and in the absence of TusA, thus making it more available for sulfur transfer under these conditions. Additionally, SufS can replace IscS in its role in providing the sulfur under certain conditions [60].

However, overexpression of IscU reduced the level of active molybdoenzymes in *E. coli* (Figure 11). When IscU is present in high amounts, it forms a complex with IscS, making it unavailable for the interaction with TusA, thus resulting in a lack of sulfur transfer for the conversion of cPMP to MPT. A similar situation was obtained when TusA was overexpressed in *E. coli* (Figure 11). Here, the levels of FeS clusters are decreased which further result in an inactive MoeA protein and thus in a decreased activity of molybdoenzymes.

Conclusively, the pleiotropic effects of a *tusA* deletion might be caused by changes in the FeS cluster concentration,

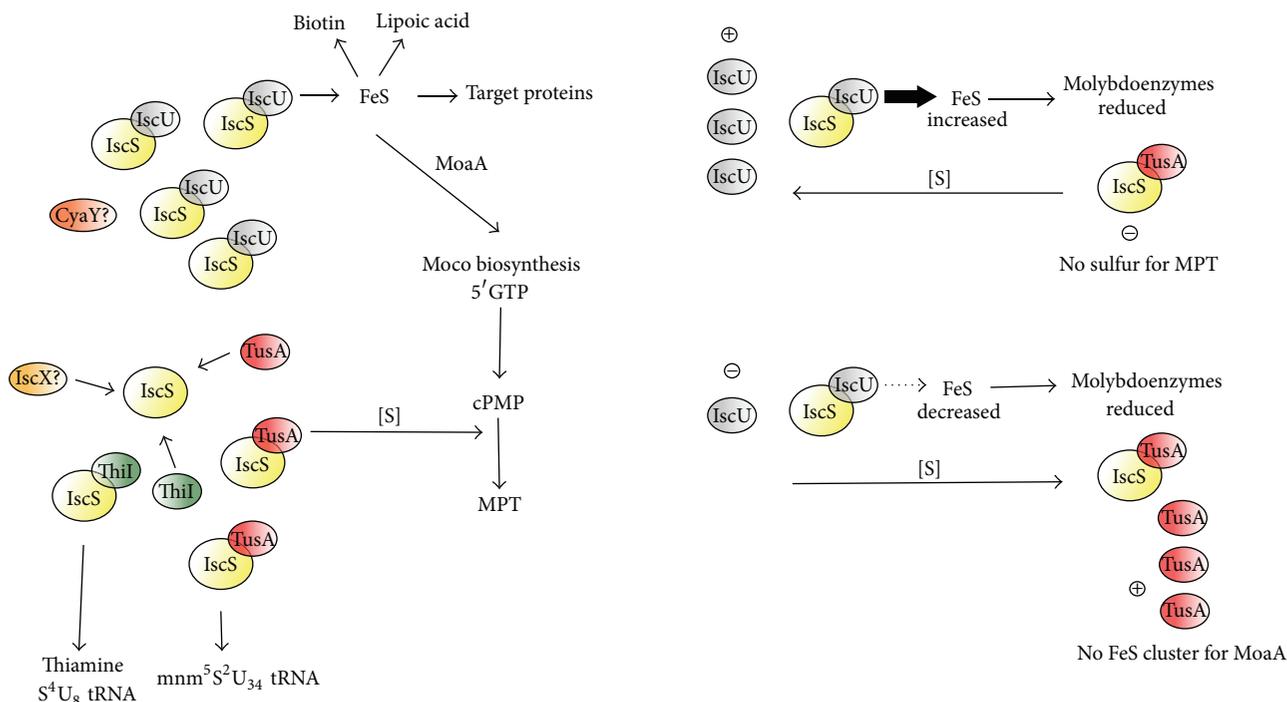


FIGURE 11: Model for the effect of elevated concentrations of TusA or IscU on Moco biosynthesis. IscS interacts with IscU, TusA, and ThiI for sulfur transfer to FeS clusters, tRNA, Moco biosynthesis, and thiamine/tRNA, respectively. FeS clusters are inserted into target proteins. Among these proteins is MoaA, involved in Moco biosynthesis (conversion of 5'GTP to cPMP). TusA is involved in the sulfur transfer for the conversion of cPMP to MPT. Increased levels of IscU reduce the level of active molybdoenzymes in *E. coli*. When IscU is present in high amounts, it forms a complex with IscS, making it unavailable for the interaction with TusA, thus, resulting in a lack of sulfur transfer for the conversion of cPMP to MPT. When the amount of TusA is increased, the levels of FeS clusters are decreased which further result in an inactive MoaA protein and thus in a decreased activity of molybdoenzymes. Detailed descriptions are given in the text.

leading to differences in the sulfur transfer to tRNA and for Moco biosynthesis. It was proposed that TusA is involved in regulating the IscS pool and shifting it away from IscU, thereby making IscS available for sulfur transfer for the biosynthesis of MPT. Additionally, major changes in the gene regulation were observed.

**3.4. The Complex Network of Sulfur Transfer to Various Biomolecules.** It seems that a lot of pathways are regulated by the availability of FeS clusters in the cell, since either they require directly FeS clusters or their synthesis is indirectly regulated by transcriptional regulators that depend on FeS clusters in the cell [138, 139]. Under these conditions when FeS clusters are required, the *isc* operon is induced and IscS then forms a complex with IscU for FeS cluster biogenesis, making it unavailable for the synthesis of other sulfur-containing biomolecules [138]. However, the other interaction partners of IscS like TusA and ThiI also direct the sulfur for important cellular processes like thiamine biosynthesis, tRNA thiolation, and Moco biosynthesis (Figure 12) [110]. It thus seems likely that, within the cell, the relative affinities of the interaction partners of IscS in addition to their concentration will determine which is the preferred partner protein that interacts with IscS. It was shown that IscU and TusA are not able to bind simultaneously to IscS, due to steric clashes of both proteins upon binding [110]. Since IscU binds

with higher affinity to IscS than TusA, this would support the synthesis of FeS clusters under sulfur limitation in the cell [125]. Since the binding sites of ThiI and TusA also overlap, this would suggest that tRNA modification for  $s^4U$  and  $s^2U$  formation is a competitive process [110].

Conclusively, changes in the concentration of the interaction partners of IscS in the cell globally affect pathways requiring sulfur. Microarray analysis of a  $\Delta tusA$  strain showed that the expression of genes regulated by FNR (*hypABCDE*, *narGHJI*, *moaABCDE*, *soxS*, *cyoABCDE*, *sdhAB*, *tdcABCDEFG*, and *feoB*) was increased [60]. The same effect was observed after overexpression of FNR in *E. coli* under aerobiosis, which led to the induction of the *isc* operon due to a higher FeS cluster demand in the cell [138]. A higher level of FeS clusters in the cell stabilizes holoFNR, thus stimulating the transcription of FNR regulated genes (like the genes for NR) [138]. Conclusively, the absence of TusA changes the level of IscS which is available for FeS cluster biosynthesis. However, a higher level of FeS clusters in the cell reduces the sulfur transfer of IscS to other biosynthetic pathways like Moco, thiamine, or thiolated tRNA. This was shown by an overexpression of the *isc* operon, which resulted in a decreased activity of molybdoenzymes [60]. The same effect was obtained after overexpression of IscU alone or the absence of TusA in the cell, which both decreased the amount of active nitrate reductase or TMAO reductase. Thus,



the availability and amount of IscS have an effect on various pathways in the cell.

Since FeS clusters are very important for cellular processes, it is conclusively suggested that one part of IscS in the cell is mostly in complex with IscU for FeS cluster biosynthesis and the other part might be available for the other interaction partners like TusA or ThiI (Figure 12). Thus, during FeS cluster formation, a portion of IscS would not be available for acceptor proteins like TusA or ThiI. When the concentration of one of the interaction partners is changed (as tested for IscU and TusA), the IscS pool is shifted to one or the other direction, with a drastic effect on gene regulation. Conclusively, our studies show that the pleiotropic effect of a *tusA* deletion might be caused by changes in the FeS cluster concentration in the cell leading to major differences in gene regulation [138]. It is proposed that TusA is involved in regulating the IscS pool and shifting it away from IscU, thereby making IscS available for sulfur transfer for the biosynthesis of MPT, thiamine, and thiolated tRNA.

However, TusA might have additional roles in the cell, since Ishii et al. [140] showed that the FtsZ-ring formation appeared to be severely impaired in *tusA*-deficient *E. coli* cells resulting in formation of a nondivided filamentous cell. These effects need to be clarified in future studies.

## Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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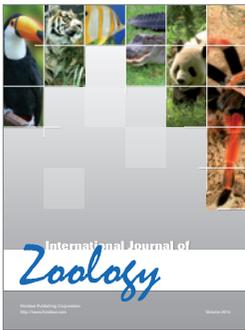
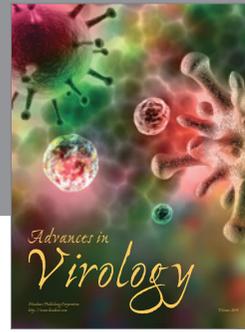
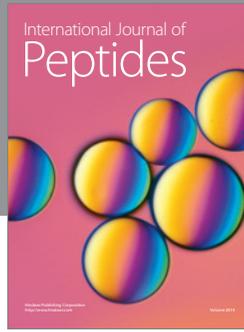
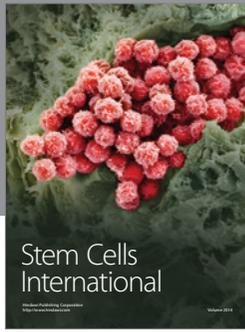
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