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Perspective

Resolving the Multidecade-Long Mystery in MoaA Radical SAM Enzyme Reveals New Opportunities to Tackle Human Health Problems

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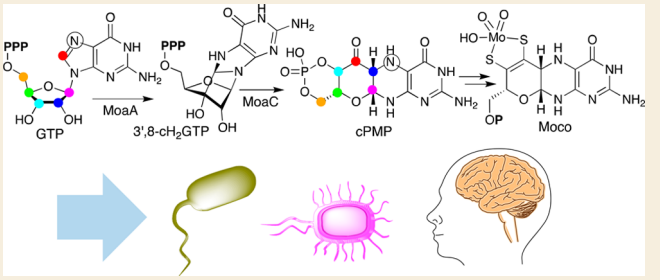
ABSTRACT: MoaA is one of the most conserved radical *S*-adenosyl-L-methionine (SAM) enzymes, and is found in most organisms in all three kingdoms of life. MoaA contributes to the biosynthesis of molybdenum cofactor (Moco), a redox enzyme cofactor used in various enzymes such as purine and sulfur catabolism in humans and anaerobic respiration in bacteria. Unlike many other cofactors, in most organisms, Moco cannot be taken up as a nutrient and requires de novo biosynthesis. Consequently, Moco biosynthesis has been linked to several human health problems, such as human Moco deficiency disease and bacterial infections. Despite the medical and biological significance, the biosynthetic mechanism of Moco's characteristic pyranopterin structure remained elusive for more than two decades. This transformation requires the actions of the MoaA radical SAM enzyme and another protein, MoaC. Recently, MoaA and MoaC functions were elucidated as a radical SAM GTP 3′,8-cyclase and cyclic pyranopterin monophosphate (cPMP) synthase, respectively. This finding resolved the key mystery in the field and revealed new opportunities in studying the enzymology and chemical biology of MoaA and MoaC to elucidate novel mechanisms in enzyme catalysis or to address unsolved questions in Moco-related human health problems. Here, we summarize the recent progress in the functional and mechanistic studies of MoaA and MoaC and discuss the field's future directions.



KEYWORDS: *radical SAM enzymes, molybdenum cofactor, biosynthesis, GTP 3′,8-cyclase, cPMP synthase, bacterial infection*

1. INTRODUCTION

MoaA is the most widely conserved radical *S*-adenosyl-L-methionine (SAM) enzyme and is found in all three domains of life. MoaA has a unique and irreplaceable function in Moco biosynthesis.[1,2] Moco is a redox enzyme cofactor found in the active site of all of the molybdate-dependent enzymes except for nitrogenases.[3] Moco mediates redox reactions frequently involving group transfers, such as oxygen atom transfer in xanthine oxidase.[3] Because of the unique catalytic capability, Moco and Moco-dependent enzymes are essential for survival or adaptation to certain conditions in most organisms. Also, Moco needs to be biosynthesized de novo by the cells that utilize Moco because Moco is chemically unstable outside the protein scaffold. Consequently, genetic mutations or pharmacological inhibition of Moco biosynthetic enzymes cause significant effects on the physiology of the organisms. In humans, Moco biosynthesis is essential for the healthy development of the brain, and genetic mutations in Moco biosynthetic enzymes cause a fatal metabolic disorder, Moco deficiency (MoCD) disease.[4] In pathogenic bacteria, Moco is essential for their adaptation to an anaerobic host environment, and pharmacological inhibition of Moco biosynthesis provides antibacterial effects.[5]

In all organisms, Moco is biosynthesized through three conserved steps (Figure 1): (1) a rearrangement of GTP into cyclic pyranopterin monophosphate (cPMP), (2) sulfur insertion to cPMP to form molybdopterin (MPT), and (3) insertion of molybdate to MPT to form Moco.[1,6] Subsequently, Moco is delivered to the active sites of Moco-dependent enzymes and receives enzyme-specific modifications such as sulfuration and nucleotidylation.[1,6] Among these biosynthetic steps, the characteristic pyranopterin structure of Moco is constructed during the formation of cPMP through the complex rearrangement of GTP. While this transformation has been known to be catalyzed by two enzymes, MoaA and MoaC, their exact functions remained elusive for more than two decades, during which many proposals were made.[7−9]

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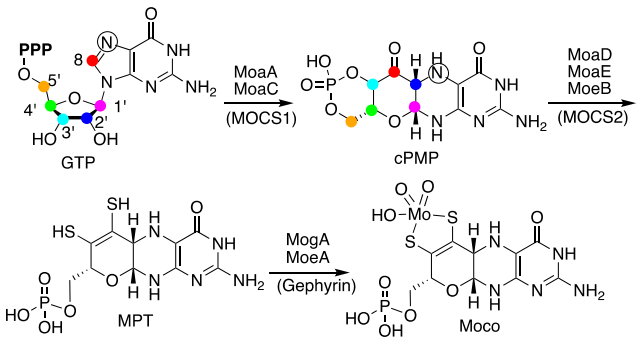

Figure 1. Conserved steps in Moco biosynthesis. Shown above arrows are bacterial Moco biosynthetic enzymes in the *moa*, *moe*, and *mog* operons. Shown in parentheses are human enzymes.

Recently, a series of biochemical and structural studies elucidated that MoaA catalyzes an unprecedented 3′,8-cyclization of GTP to produce a labile precursor, 3′,8-cyclo-7,8-dihydroguanosine 5′-triphosphate (3′,8-cH$_2$GTP), and MoaC catalyzes the complex rearrangement of 3′,8-cH$_2$GTP into cPMP.[2,10,11] The functional characterization of MoaA and MoaC provided the basis for the detailed characterization of their unique catalytic mechanisms. MoaA was a founding member of the radical SAM superfamily when it was first defined by Sofia et al.[12] It is also a representative member of the SPASM-twitch family of radical SAM enzymes with multiple 4Fe-4S clusters.[13] Also, the MoaA-catalyzed 3′,8-cyclization is unprecedented and does not proceed without assistance from the enzyme active site environment.[14] Thus, MoaA serves as an excellent model system to study the catalytic function of 4Fe-4S clusters in the SPASM-twitch family and the mechanism by which radical SAM enzymes catalyze free-radical-mediated reactions. Also, the MoaC-catalyzed reaction is one of the most complex rearrangement reactions in enzyme catalysis,[11] and its understanding will provide insights into the mechanism by which enzymes catalyze complex rearrangement reactions. Furthermore, since Moco biosynthesis has been linked to human health problems,[4,5] mechanistic understandings in MoaA and MoaC catalysis will form a critical basis to solve such problems. Therefore, in this perspective, we will summarize the current status of our understanding of the functions and mechanisms of MoaA and MoaC and the potential future directions of the field.

2. BIOLOGY OF Moco AND ITS BIOSYNTHESIS

2.1. Introduction

Moco is a redox enzyme cofactor consisting of molybdate coordinated by a pyranopterin dithiolene ligand called molybdopterin and mediates redox reactions via its redox-active molybdate center.[3] In some organisms, tungstate is used instead of molybdate (tungsten cofactor, W-co). Most of the reactions catalyzed by Moco- (and W-co)-dependent enzymes are oxygen atom transfers between the substrate and the solvent water that involve changes of the redox state of the carbon or heteroatom of the substrate to which the oxygen is attached. The reactions catalyzed by Moco-dependent enzymes are unique compared to other redox enzymes and frequently play pivotal roles in metabolic and catabolic pathways. Consequently, Moco or W-co is found in almost all organisms in all kingdoms of life and is frequently essential for their lives or adaptation to a certain environment.

In most organisms, Moco requires de novo biosynthesis in the cells that express Moco-dependent enzymes because of the limited chemical stability of Moco outside the protein scaffold. The only exception known so far is *Caenorhabditis elegans* reported to take up Moco from its bait microorganisms.[15] Although Moco-bound proteins, and not free Moco, were proposed to be taken up as the source of Moco,[16] the mechanism of such a salvage pathway is not understood. In all of the other organisms, Moco biosynthesis is essential for the production of functional Moco-dependent enzymes. Consequently, perturbations in Moco biosynthesis by genetic mutation or pharmacological inhibition results in the pleiotropic loss of all the Moco-dependent enzyme activities. Here, we focus on such effects in humans and pathogenic bacteria as they are both related to human health problems and require more development.

2.2. Moco in Humans

In humans, Moco-dependent enzymes are found in catabolic and detoxification pathways. Perhaps one of the best characterized Moco-dependent enzymes in humans is a xanthine oxidase (XO) that catalyzes the oxidation of hypoxanthine into xanthine and then into uric acid during the purine nucleotide catabolism.[17] When the uric acid accumulates in the blood, it forms sharp needle-like crystals in joints, tendons, and surrounding tissues and causes gout characterized by acute and chronic pain. Since XO is the rate-determining step of uric acid formation, pharmacological inhibition of XO lowers the uric acid level and is a proven approach to gout treatment.

On the other hand, a pleiotropic loss of all the activities of Moco-dependent enzymes by genetic mutations in Moco biosynthetic genes in humans causes a fatal disease, Moco deficiency (MoCD),[18] characterized by seizures, progressive neurological symptoms, and impaired brain development. Although the mechanism of neurological impairment is not fully understood, the most severe symptoms are caused by the lack of Moco-dependent sulfite oxidase activity, which results in the accumulation of toxic sulfur metabolites, such as *S*-sulfocysteine that constitutively activate the *N*-methyl-D-aspartate receptor and causes a brain damage.[4] Because the early deaths of the patients limit the inheritance of this disease, the reported cases of MoCD are <1 in 100,000, but a significant number is thought to be misdiagnosed.[6] Currently, MoCD is incurable, and the most frequent consequence of the disease is death within a year of birth. Experimental therapy of daily injection of a biosynthetic intermediate (cPMP, Figure 1) has been shown to significantly reduce the neurological symptoms of eight out of 11 patients with Type A MoCD.[19]

2.3. Moco in Pathogenic Bacteria

In some important pathogenic bacteria, such as *Mycobacterium tuberculosis* (*Mtb*), *Pseudomonas aeruginosa*, and pathogenic *Escherichia coli*, the critical roles of Moco in their virulence have been reported based mostly on knockout studies of Moco biosynthetic genes.[5] While the mechanistic causes of the decreased virulence remain primarily uncharacterized, it is thought that Moco is essential for these bacteria to adapt to environments where oxygen is not readily available as the electron acceptor for respiration. In addition, some of these pathogens harbor multiple copies of Moco biosynthetic genes with partially redundant functions, making it difficult to

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understand the role of Moco in their pathogenesis. Since the role of Moco in bacterial virulence has been comprehensively reviewed recently,[5] we will briefly summarize those known for the representative pathogens and gut microbiome.

In *Mtb*, several lines of evidence provided strong support for the critical role of Moco in pathogenesis.[20] First, the presence of multiple copies of Moco biosynthesis genes suggests the significance of Moco in *Mtb* physiology.[20,21] Intriguingly, many other mycobacteria with significantly diminished virulence, such as *M. smegmatis*, carry only a single set of Moco biosynthetic genes. Acquisition of multiple Moco biosynthetic gene clusters has been proposed as a process of *Mtb* evolution toward pathogenicity.[22] Second, the knockout of one of the Moco biosynthetic genes responsible for cPMP or MPT formation causes significant loss of virulence.[20,23−28] The partially overlapped function of the multiple copies of Moco biosynthetic genes has been suggested based on the attenuated and not a complete loss of the production of Moco in the knockout of one of the copies.[20] Unfortunately, the reported knockout studies are part of large-scale transposon screening of virulence factors, and the detailed mechanism of the attenuated virulence is not known.[20] Simultaneous knockout of all the genes for a particular Moco biosynthesis step has not been reported. Therefore, it is currently unknown whether Moco is essential for *Mtb* survival. Third, an anti-*Mtb* compound, TCA1, exhibits the activity against nonreplicating *Mtb* by inhibiting MoeW and the Moco biosynthesis.[29] MoeW is a homologue of MoeB, a sulfurtransferase for MPT synthase (Figure 1), and is uniquely found in *Mtb* and *Mycobacterium bovis* and not in other mycobacteria.[29] To our knowledge, no functional characterization of MoeW has been reported. Nevertheless, TCA1 reduced the amount of Moco in *Mtb*, which was attributed to the ability of this compound to exhibit antibiotic activity against nonreplicating *Mtb*.

Although the mechanism of the reduced pathogenesis by decreased production of Moco remains ambiguous, Moco-dependent nitrate reductase (NR) is thought to play a crucial role in virulence. NR is essential for *Mtb* to fit and survive under a low-oxygen environment, the condition assumed in the host body.[30] In fact, NR activity was shown essential to persist in the host lungs of a guinea pig infection model.[31] The significance of NR in *Mtb* physiology is also demonstrated by the ability to clinically detect *Mtb* using colorimetric NR assay.[32] Several other Moco-dependent enzymes, including rotenone-sensitive type I NADH dehydrogenase and CO dehydrogenase, have been shown to be involved in *Mtb* pathogenesis. Therefore, a pleiotropic loss of Moco in *Mtb* by inhibition of the biosynthetic pathway would significantly impact the physiology and/or virulence of *Mtb*.

In *P. aeruginosa*, a mutation in the Moco-dependent nitrate reductase (NarG) led to growth defects in cystic fibrosis sputum medium, reduced swarming ability in vitro, reduced biofilm formation, attenuated survival in biofilm, and decreased virulence in *C. elegans* infections.[33,34] Consistent with the significance of NarG, a mutation in a sulfide carrier protein (PA1006) involved in the MPT biosynthesis significantly diminished the virulence and biofilm formation.[35,36] While the amount of Moco production was not quantified, the involvement of PA1006 in Moco biosynthesis was supported by the extensive in vivo interaction of this protein with the other Moco biosynthesis enzymes and the significantly reduced level of nitrate assimilation in the PA1006 gene knockout strain.[35] Still, the mutation in PA1006 unlikely resulted in the complete loss of Moco production considering the presence of multiple sulfide carrier proteins in *P. aeruginosa* and their partial functional redundancy. Therefore, similar to *Mtb*, simultaneous knockout of all copies of Moco biosynthetic genes for either cPMP or MPT synthesis is required to investigate the impact of Moco in the pathogenesis of *P. aeruginosa*.

Anaerobic respiration and metabolisms have unique and likely critical functions in the community of microbiome, especially in the anaerobic or microaerobic conditions in the human gut. Under such oxygen-limiting conditions, Moco and Moco-dependent enzymes could play key roles. In fact, metagenomic sequencing of gut microbiome revealed that Moco-dependent enzyme genes as a signature of gut inflammation-associated dysbiosis.[37] Consequently, inhibition of Moco biosynthesis using tungstate prevented the dysbiotic expansion of Enterobacteriaceae during gut inflammation and significantly reduced the severity of inflammation.[38]

While the above-described organisms represent the best-characterized examples in terms of the effects of Moco on virulence and pathogenesis, this is also likely the case in many other pathogens. For example, in *Burkholderia thailandensis* (genetically close to pathogenetic *Burkholderia pseudomallei*), transposon mutation of the *moeA* gene required for the molybdate insertion shows reduced biofilm formation and motility, which were highly correlated with the reduction of nitrate reductase activity.[39] In general, in many bacteria, Moco is likely required for adaptation to the low O_2 environment that they experience in hosts. Therefore, understanding the role of Moco in pathogenic bacteria is important to improve our understanding of the mechanism by which these pathogens adapt to different environments frequently distinct from the lab culture conditions.

3. Moco BIOSYNTHESIS AND MoaA AND MoaC FUNCTIONS

3.1. Functions of MoaA and MoaC in cPMP Biosynthesis

In all organisms, the characteristic pyranopterin structure of Moco is biosynthesized during the transformation of GTP into cPMP.[1,6] cPMP was originally identified in the 1990s through pioneering studies by the Rajagopalan lab using *E. coli* strains with mutations in the *moaD* gene encoding the small subunit of MPT synthase.[40] The same compound was also identified in urine samples of human MoCD patients carrying mutations in the *moaD* homologue gene (MOCS2).[41] The involvement of MoaA and MoaC in the cPMP formation was established through the characterization of *E. coli* with genetic mutations that disrupt Moco and cPMP production. These functional assignments were confirmed later in the 2000s by demonstrating the in vitro transformation of GTP into cPMP by recombinant MoaA and MoaC.[42] However, the specific functions of these two enzymes/proteins remained elusive.

The mechanism of transformation of GTP into cPMP has attracted significant scientific interest. Early isotope incorporation studies by the Rajagopalan lab revealed that the C-8 of the guanine base was incorporated specifically into the C-12 of cPMP[43] (Figure 1). Together with the studies with other ^{13}C or ^{15}N-labeled guanosine isotopologs,[44] the results suggested that C8 of guanine base of GTP between C2′ and C3′ of ribose[43] (Figure 1). These observations were in sharp contrast to all the other pterin-related biosynthetic pathways, such as those for folate, biopterin, and riboflavin, in which the C-8 of

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guanine is hydrolyzed and released as formate by the action of GTP cyclohydrolase and is not retained in the final metabolite[45−47] (Figure 2). Therefore, these results suggested the unique mechanism of pterin ring formation in the Moco biosynthesis.

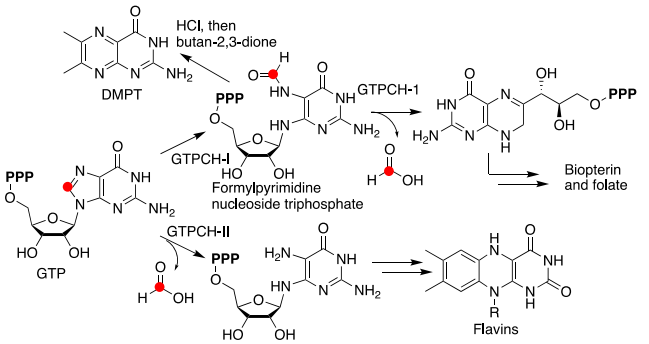

Figure 2. Fates of C8 of GTP in biopterin, folate, and flavin biosynthesis. In these pathways, GTP cyclohydrolase I or II (GTPCH-I and -II) hydrolyze and release C8 of GTP as formic acid.

The functions of MoaA and MoaC were studied by multiple groups, and many functional proposals have been made. The functional characterization of MoaA was facilitated by its annotation as a radical SAM enzyme by Sofia et al.[12] Together with the notion that radical SAM enzymes catalyze chemically unique transformations, in the mid-2000s, MoaA was first assumed to catalyze the majority or all of the complex rearrangement reaction between GTP and cPMP. In the 2000s, Schindelin et al. reported the activity of recombinant MoaA and MoaC to transform GTP into cPMP. MoaA was found to bind GTP, which was used as evidence that MoaA catalyzes the complex rearrangement of GTP.[48] Schindelin et al. also mentioned in their publication[48] that an incubation of MoaA with GTP and SAM in the absence of MoaC produced a compound that can be converted into dimethylpterin upon treatments with acid followed by butane-2,3-dione. This observation was compared to those made for GTPCH-I (Figure 2), which catalyze a complex rearrangement of GTP into dihydroneopterin triphosphate during the folate biosynthesis.[45,49] The GTPCH-I catalysis proceeds through hydrolysis of the N9−C8 bond of the guanine base of GTP with formylpyrimidine nucleoside triphosphate as a reaction intermediate.[45,46] This intermediate can be derivatized to dimethylpterin upon treatments with acid followed by butane-2,3-dione[48] (Figure 2). Since the MoaA assays without MoaC also accumulated a compound that can be derivatized to dimethylpterin, MoaA catalysis was proposed to proceed through the same intermediate[48] (Figure 3A).

On the other hand, the function of MoaC remained ambiguous. Various possibilities for the role of MoaC were discussed, including MoaC as a regulatory subunit of MoaA.[42] However, no evidence for a strong interaction between MoaA and MoaC was found. Instead, although data were never published, there was a strong belief in the field that the MoaA assay in the absence of MoaC does not produce pyrophosphate, and thus, MoaC is involved in the cyclic phosphate formation.[1] Despite the lack of published data, by the late 2000s, a majority of the field assumed that MoaC is responsible only for cyclic phosphate formation (Figure 3A). In this notion, MoaA catalyzes the complex rearrangement of GTP into pyranopterin triphosphate with formylpyrimidine nucleotide as an intermediate, while MoaC catalyzes only the cyclic phosphate formation and is not involved in the rearrangement.

Under these notions, it was surprising when MoaA product was characterized for the first time as 3′,8-cH$_2$GTP[2] (Figure 3B). Characterization of MoaA product had been hampered by its limited chemical stability at acidic pH or in the presence of oxygen and the limited amount produced by MoaA due to the apparent strong product inhibition. The ability to produce MoaA in a gram quantity coupled with the improved anaerobic techniques allowed the isolation and structural characterization of 3′,8-cH$_2$GTP.

The relevance of 3′,8-cH$_2$GTP to Moco biosynthesis was demonstrated through in vitro and in vivo assays and X-ray crystallography. The purified 3′,8-cH$_2$GTP was specifically recognized by bacterial (*E. coli*[11] or *Staphylococcus aureus*[2]) MoaC as well as human MoaC homologue,[2] MOCS1B, with K_m values of <0.060−0.25 and 0.79 μM, respectively, suggesting that the functions of MoaC are conserved among bacteria and humans and likely in other organisms as well. Subsequently, the structures of *E. coli* MoaC in complex with 3′,8-cH$_2$GTP or cPMP were solved.[11] In these crystal structures,[11] 3′,8-cH$_2$GTP and cPMP were bound to the

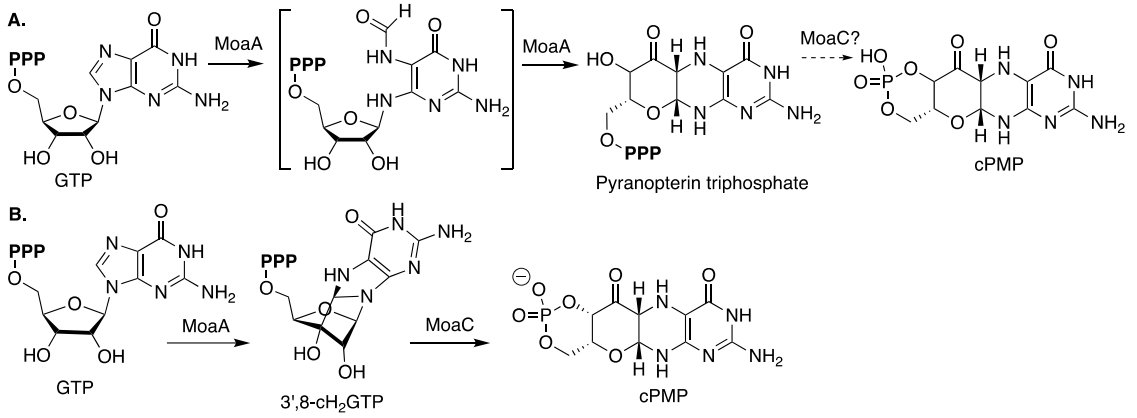

Figure 3. Proposed functions of MoaA and MoaC. (A) Early proposals for MoaA being responsible for the complex rearrangement of GTP into pyranopterin triphosphate with ambiguous function for MoaC. (B) Revised functions of MoaA and MoaC, where MoaA is responsible for the GTP 3′,8-cyclization, and MoaC catalyzes the complex rearrangement reaction and constructs the cPMP structure.

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previously proposed[50] ligand-binding pocket. Both compounds were interacting with the same set of amino acid residues, all of which were critical for in vitro and in vivo catalytic function of MoaC, suggesting that this pocket is likely the active site of MoaC. These combined biochemical and structural characterizations eventually revised the functions of MoaA and MoaC with 3′,8-cH$_2$GTP as the physiological product of MoaA and substrate of MoaC.

3.2. Unsolved Questions in Bacterial cPMP Biosynthesis

Considering the conservation of the catalytic residues in MoaA and MoaC, their general functions are likely conserved in all organisms, although details of their mechanisms could be different. Likewise, although no functional characterizations have been reported, the same catalytic functions are expected for the multiple copies of MoaA and MoaC in pathogenic bacteria described above. However, between the different copies of MoaA/MoaC in these pathogens, the functional redundancy appears only partial, and each copy likely has a specific biological function. They may be differently regulated to ensure Moco production in various growth conditions.[22] Alternatively, each set of Moco biosynthetic enzymes may form a weakly interacting complex to ensure the delivery of chemically labile intermediates, such as 3′,8-cH$_2$GTP. The in vivo interactions among MoaA, MoaC, MPT synthase, and sulfur trafficking enzymes have been reported in *P. aeruginosa* based on GFP protein fragment complementation assay[35] (GFP-PFCA). Although the details of the nature and consequence of such interactions are currently unknown, these observations may suggest the presence of a mechanism for the efficient flux of Moco biosynthesis in the crowded cellular environment. Clustering of metabolic enzymes has been emerging mostly in eukaryotic cells, represented by purinosome,[51,52] where chemically labile intermediates must be efficiently transferred between biosynthetic enzymes. Similar mechanisms may exist in Moco biosynthesis.

3.3. cPMP Formation in Humans

In humans, Moco biosynthesis has additional aspects in the expression of splice variants and compartmentalized biosynthesis, which may be related to the regulation of the pathway. Human homologues of MoaA and MoaC are encoded in a single gene, MOCS1, as splice variants, MOCS1A and MOCS1AB (Figure 4A). MOCS1A is the MoaA homologue and is expressed as two major splice variants, the Larin and Reiss variants[53] (Figure 4B). These variants differ by the N-terminal amino acid sequence transcribed from exon 1a and 1b, respectively. The cellular localization study suggested that exon 1a is required for mitochondrial translocation, and variants with exon 1a are localized in the mitochondria matrix.[54] On the other hand, variants lacking exon 1a were found in the cytosol. Together with the localization of MOCS1B in mitochondria, the Larin variant with exon 1a is likely responsible for Moco biosynthesis. The role of Reiss and other variants lacking exon 1a in Moco biosynthesis is unknown.

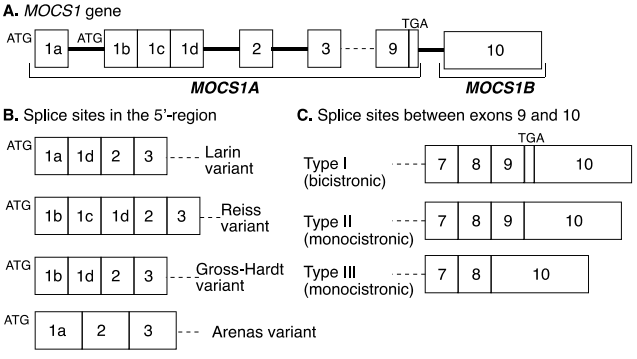

Figure 4. MOCS1 gene splice variants in humans. (A) Schematic representation of exons coding the *MOCS1* gene and the intervening introns. (B) Reported splice sites in the 5′-region of the *MOCS1* gene. (C) Reported splice sites between exons 9 and 10 of the *MOCS1* gene.

MOCS1AB is expressed by skipping the translation of the stop codon (type II variant) or splicing out the exon 9 that encodes the stop codon (type III variant, Figure 4C). Consequently, MOCS1AB is missing the catalytically essential C-terminal Gly residues (GG motif; see sections 4.2 and 6.1), which are conserved among all MoaA homologues in bacteria and eukaryotes. Thus, MOCS1AB is thought to exhibit only the MoaC function. Recently, an exon 1a independent delivery of MOCS1AB to the mitochondria was proposed based on fluorescence microscopy and cell fractionation experiments.[54] In this model, MOCS1AB is expressed in the cytosol, followed by proteolytic cleavage between MOCS1A and MOCS1B on the surface of the mitochondrial outer membrane. The resulting MOCS1B protein is transported across the mitochondrial membrane and delivered to the mitochondria matrix. Based on this model, MOCS1A and MOCS1B are both localized in the mitochondria matrix and catalyze the transformation of GTP into cPMP. Since MPT synthase is localized in the cytosol, cPMP likely passively diffuses through the mitochondria membrane to the cytosol. Whether MOCS1A and MOCS1B interact with each other is currently unknown.

3.4. Physiological Reductant of MoaA and MOCS1A

Radical SAM enzymes require a reductant to reduce their 4Fe-4S clusters. In general, the physiological reductant of radical SAM enzymes is poorly understood. Therefore, in vitro characterizations are frequently performed with chemical reductants, such as sodium dithionite. While these chemical reductants are frequently sufficient for in vitro functional and mechanistic studies, they sometimes place the 4Fe-4S clusters or the enzymes into catalytically irrelevant (redox) states. In fact, in some cases, such as PqqE[55] and NosL,[56] the use of chemical reductants leads to abortive SAM cleavage or the alteration of regiospecificity,[57] highlighting the significance of understanding the physiological reductant. Understanding the physiological reductase is also important to understand the physiological function of radical SAM enzymes and their catalytic efficiency in vivo.

Despite the benefits of understanding the physiological reductase of radical SAM enzymes, such a reductase system is not known for most radical SAM enzymes. Currently, in bacteria, the only reported physiological reductant of radical SAM enzymes is flavodoxin (FldA) for NrdH, the radical SAM activase for the class III ribonucleotide reductase[58] (NrdG). However, many other bacteria do not carry close homologues for FldA, and therefore the physiological reductant for bacterial radical SAM enzymes, including MoaA, remains elusive.

In eukaryotes, a specific reduction system has been identified for Dph1-Dph2, a noncanonical radical SAM enzyme complex responsible for the formation of diphthamide, a highly conserved post-translational modification on eukaryotic

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elongation factor 2 (eEF2).[59] Dph1-Dph2 catalyzes reductive cleavage of SAM to transiently generate 3-amino-3-carboxypropyl (ACP) radical that adds to the His residue of eEF2 (Figure 5A). In yeast, the Dph1-Dph2 catalysis requires an

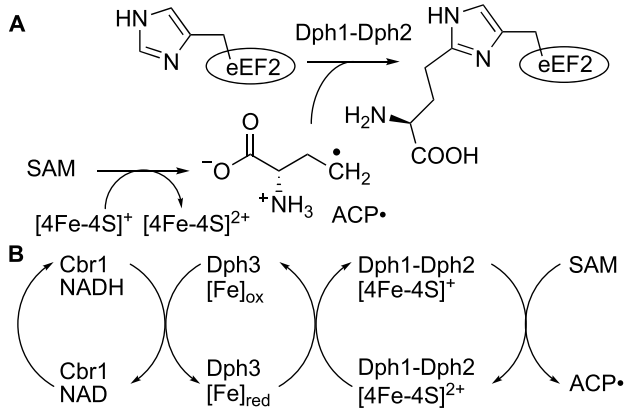

Figure 5. (A) Reaction catalyzed by Dph1-Dph2. (B) Electron chain for the reduction of Dph1-Dph2.

electron chain formed by Cbr1[60] and Dph3[61] (Figure 5B). Cbr1 is a transmembrane NADH cytochrome b_5 reductase embedded in the endoplasmic reticulum membrane and mitochondrion outer membrane, with the catalytic domain facing the cytosol. Cbr1 oxidizes NADH into NAD^+ and transfers the electrons to Dph3, a cytosolic iron protein that donates the electron specifically to the Dph1-Dph2 complex. Cbr1 homologues, including mitochondrial cytochrome b_5 reductase Mcr1, were also shown to serve as reductases for Dph1-Dph2. Also, Cbr1 and its homologues were shown to serve as reductases for Elp3,[60] a canonical radical SAM enzyme responsible for the 5-carboxymethyluridine (cm^5U) formation during tRNA wobble uridine modification.[62] These studies suggest the redundancy of the reductases and their functions, potentially complicating the identification of the physiological reductant of radical SAM enzymes in general. Cbr1 is unlikely the reductase for MOCS1A because cPMP formation is thought to proceed in the mitochondria matrix (see section 3.3). Thus, the reductase for MOCS1A is currently unknown. Identification and characterization of the physiological reductant could reveal the potential mechanism by which MoaA/MOCS1A activity is regulated in vivo.

4. MoaA CATALYTIC MECHANISMS

4.1. Overview

The successful functional characterization of MoaA and MoaC provided critical foundations for mechanistic characterization of MoaA and MoaC catalysis. In particular, MoaA serves as a model system to understand how a radical SAM enzyme catalyzes difficult radical reactions. Initial isotope tracing experiments and the stoichiometry of the reaction suggested that MoaA uses 5′-dA• to abstract the H-3′ of GTP to generate GTP C3′ radical that is then added to C8 of guanine base (Figure 6).[2] The resulting aminyl radical would then be reduced by a transfer of a proton and an electron. As discussed below, the 3′,8-cyclization of purine nucleoside/nucleotide was unprecedented, and the aminyl radical reduction requires a specific mechanism with a strong reductant. Also, considering the limited chemical stability of 3′,8-cH_2GTP, the MoaA active site has to be nonacidic and nonoxidative. Therefore, MoaA must be furnished with all the tricks that meet all these requirements.

The 3′,8-cyclization of purine nucleoside/nucleotide was unprecedented in biological or chemical reactions. Formation of the radical at the 3′-position of deoxynucleotides and nucleotides has been reported for several synthetic and biological reactions. Perhaps the best characterized is the reaction catalyzed by ribonucleotide reductase (RNR) that catalyzes the reduction of ribonucleotides into deoxyribonucleotides via C3′ radical intermediate.[63−65] Perturbation of this reaction by mutations in the active site residues or by using substrate analogues frequently results in dissociation of the base.[66−68] In DNA, the formation of the C3′ radical is thought to cause strand scission and base dissociation.[69,70] In none of the reported reactions, the addition of C3′• to C8 of purine base has been observed. Therefore, the MoaA active site must be furnished to specifically catalyze the 3′,8-cyclization of GTP.

The reductive quenching of aminyl radical also requires specific mechanisms. In DNA, 5′,8-cyclization of purine nucleotides have been reported as products of photo damage.[71] However, these reactions usually yield oxidized products. Radical addition to aromatic systems is also known in many other radical SAM enzymes.[57] However, in all such reported cases, the product radical after the radical addition is oxidatively quenched. Examples of such reactions include the C8 methylation of adenosine nucleotide by Cfr and RlmN,[72] and cross-linking reactions of ribosomally synthesized peptides by PqqE[55] and StrB.[73] Oxidative quenching in these reactions is likely facilitated by aromatization of the product and the presence of a 4Fe-4S cluster as an electron acceptor.[57,74] In contrast, MoaA specifically reduces the 3′,8-cGTP-N7•

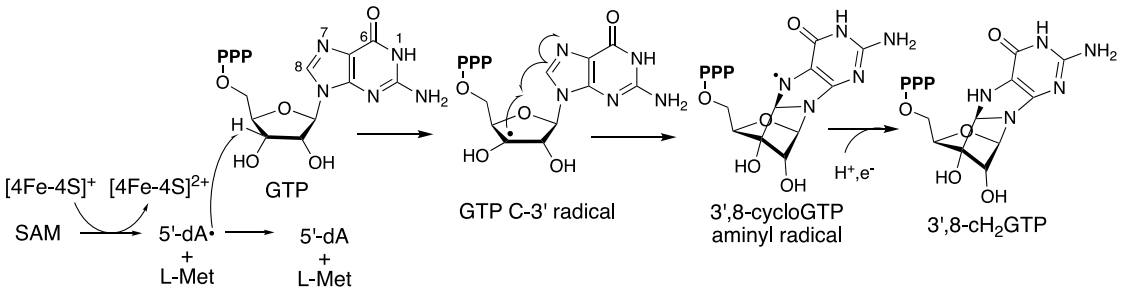

Figure 6. Overview of the MoaA-catalyzed GTP 3′,8-cyclization.

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intermediate and produces 3′,8-cH$_2$GTP. No oxidatively quenched and aromatized product is detectable. Therefore, there must be a mechanism in MoaA to specifically reduce the aminyl radical.

4.2. Structure of MoaA

MoaA is a representative member of the SPASM-twitch family, the largest group of radical SAM enzymes with one or more auxiliary 4Fe-4S clusters.[13,75] MoaA harbors two 4Fe-4S clusters (Figure 7); the canonical radical SAM 4Fe-4S cluster

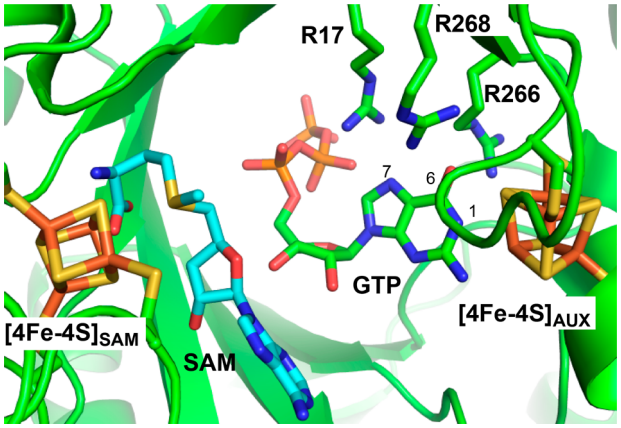

Figure 7. Structural model of MoaA active site created by overlaying the reported structures of MoaA in complex with SAM[42] (PDB ID: 1TV8, cyan) and with GTP[48] (PDB ID: 2FB3, green).

(RS cluster) and an auxiliary (AUX) cluster. The AUX cluster of MoaA is coordinated by three Cys residues, and the fourth ligand is N1 of GTP's guanine base. The GTP-binding site is characterized by the presence of three conserved Arg residues (17, 266, and 268; numberings based on *S. aureus* MoaA). These Arg residues interact with the guanine base; R17 is close to guanine N7, and R266 and R268 are in H-bond distances from guanine O6. On the other hand, the bottom of the active site consists of hydrophobic amino acid residues, and the ribose moiety of GTP does not form H-bond interactions with any of the active site residue. As discussed below, these active site architectures likely provide a specific environment to catalyze the unique 3′,8-cyclization of GTP.

The crystal structures of MoaA in complex with either SAM[42] or GTP[48] have been reported. However, no structure is currently available with both SAM and GTP. Also, in all the reported MoaA structures, the C-terminal 11 amino acid residues are disordered and not detectable. The C-terminus of MoaA has two strictly conserved Gly residues (GG motif). Mutations of these residues to any other amino acids, including Ala, completely abolish the activity of bacterial MoaA,[76] and mutations of corresponding residues in human MOCS1A cause MoCD disease.[41,77] The active site of MoaA in the reported crystal structures is highly exposed to solvent. In general, the active sites of many other radical SAM enzymes are very well isolated from the external environment and provide the inert environment for radical reactions to proceed without significant side reactions.[78] Thus, it is likely that a significant portion of the MoaA active site has eluded characterization. This missing part is likely the C-terminal tail that is disordered in the crystal structure but essential for the catalytic activity of MoaA.[76] Therefore, further structural characterization is required to understand the complete structure of the MoaA active site.

4.3. Recent Advancement in Mechanistic Understanding of MoaA

Recent mechanistic studies have started to illuminate the mechanism that allows MoaA to catalyze the otherwise difficult C3′• addition to C8.[14] This study combined a comprehensive kinetic characterization and density functional theory (DFT) computations of the MoaA-catalyzed 3′,8-cyclization reaction. Initially, the kinetic study revealed the presence of a shunt pathway (Figure 8, path B) that yields (4′*S*)5′-deoxyadenosine (4′*S*-5′-dA) via 5′-deoxyadenos-4′-yl radical (5′-dA-C4′•). Kinetic comparison of the normal and shunt pathways (Figure 8, paths A vs B) allowed the determination of the rate constant for the C3′-C8 bond formation. Together with DFT

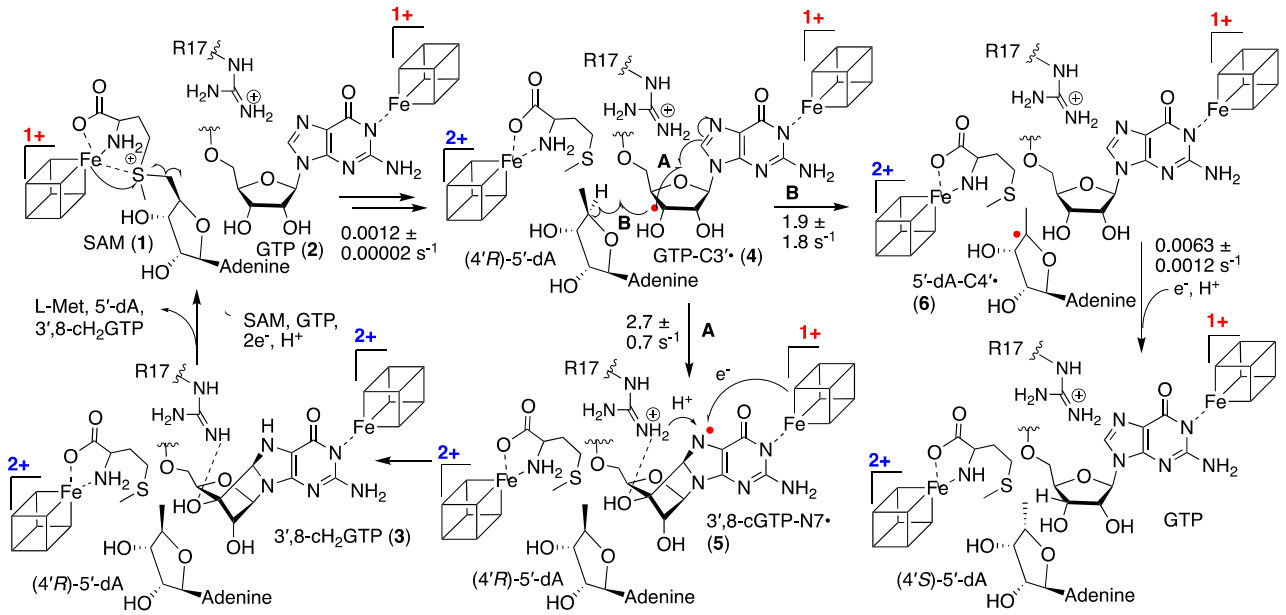

Figure 8. Proposed mechanism of MoaA catalysis. This figure was adapted from ref 79. Copyright 2021 American Chemical Society.

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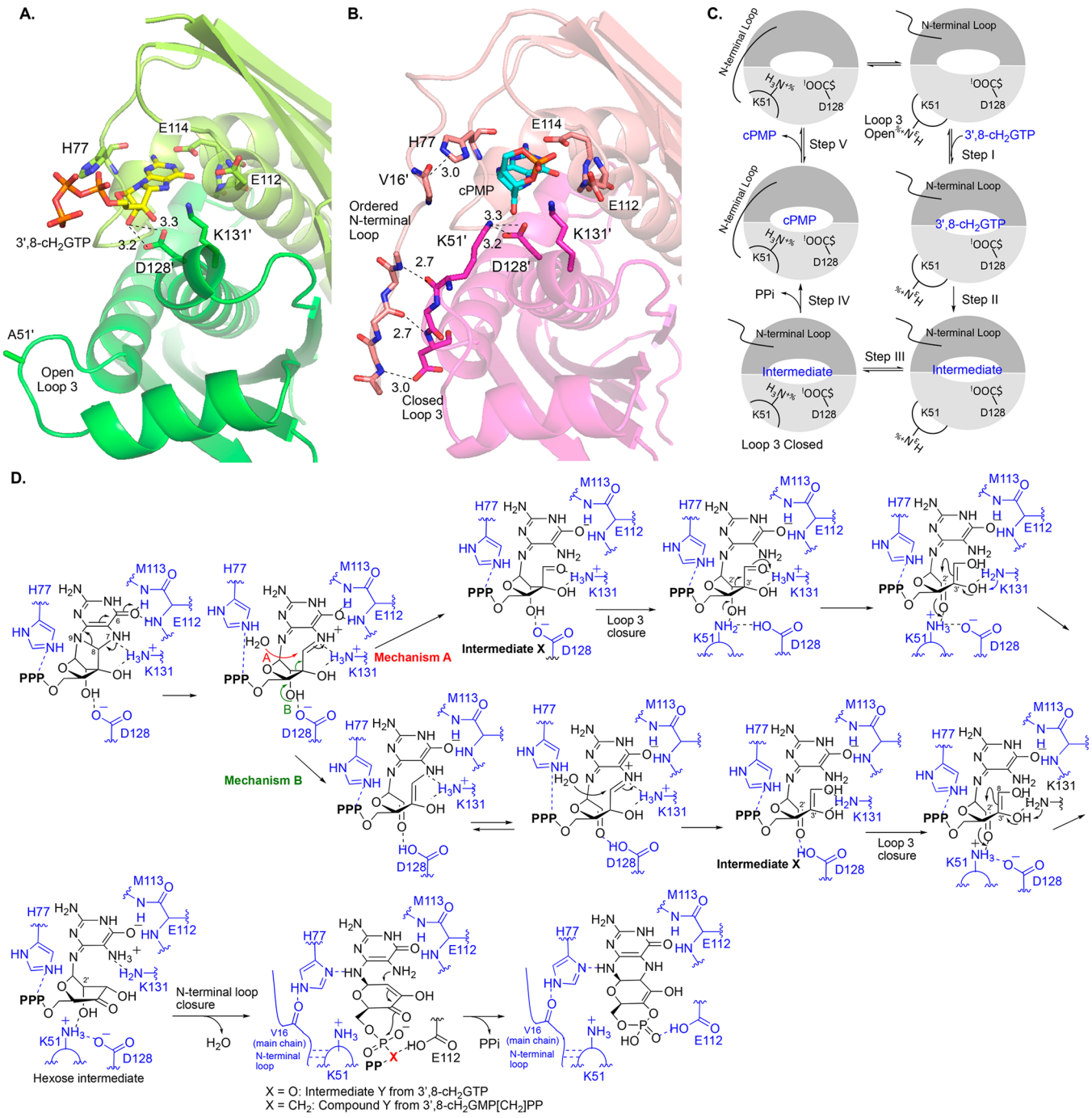

Figure 9. Structure and mechanism of MoaC. (A,B) Crystal structures of K51A-MoaC in complex with 3′,8-cH$_2$GTP (A) and wt MoaC in complex with cPMP (B). (C) Proposed conformationally guided transformation of 3′,8-cH$_2$GTP into cPMP. (D) Proposed biochemical mechanism of the 3′,8-cH$_2$GTP rearrangement into cPMP. Figures were adapted with permission from ref 11. Copyright 2015 Proceedings of the National Academy of Science.

computations, the study suggested that MoaA accelerates the C3′• addition to C8 by 6−9 orders of magnitude by restricting the GTP conformation and stabilizing the transition state through H-bond interaction between 3′-OH and R17. This study represented the first evidence in radical SAM enzymes where transition state stabilization is used as the mechanism of rate acceleration.

More recently, the catalytic function of the auxiliary 4Fe-4S cluster was probed using a combination of protein film voltammogram, electron paramagnetic resonance (EPR), and DFT computations.[79] The study revealed the reduction potentials of the RS and AUX clusters as 510 and 455 mV, respectively. Furthermore, Q-band EPR characterization of the 5′-dA-C4′• demonstrated its exchange interaction with the AUX cluster in the reduced state. The large exchange coupling constant (263 MHz) was unexpected as the 5′-dA-C4′• is >10 Å separated from the AUX cluster and suggested a super-exchange pathway through the guanine base of GTP. Therefore, the observation provided the first experimental evidence that during the catalytic turnover, the AUX cluster is in the paramagnetic and reduced 1+ state and electronically coupled to the guanine base of GTP. Together with DFT

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computations, the observed reduction potential of the AUX cluster is most consistent with the aminyl radical reduction by a proton-coupled electron transfer mechanism with the R17 residue as the proton donor and the AUX cluster as the electron donor.

The observed reduction potentials of MoaA RS and AUX clusters are unique compared to those of other SPASM-twitch family members that catalyze oxidative radical quenching. In the SPASM-twitch members that catalyze oxidative radical quenching, such as SCIFF maturase,[80] MftC,[81] and SuiB,[82] the reduction potentials of RS clusters are more positive than those of AUX clusters, allowing the reduction of the RS cluster without reducing the AUX cluster. On the other hand, in MoaA, the AUX cluster is more positive than the RS cluster, which ensures that all of the AUX cluster is reduced under the condition that the RS cluster is reduced. Therefore, the reduction potentials of RS and AUX clusters are likely finely tuned based on the redox chemistry catalyzed by the enzyme.

4.4. Unsolved Questions

First and foremost, the catalytically relevant active site structure remains elusive. As described above, the C-terminal tail is disordered and not modeled in the reported crystal structures. Therefore, although the C-terminal tail likely forms part of the active site, it is unknown how the C-terminal tail binds to the active site and whether it provides an additional mechanism to catalyze the 3′,8-cH$_2$GTP formation. Also, mutations in the GG motif in MOCS1A cause human MoCD disease. The C-terminal tail amino acid sequence other than the GG motif varies significantly among different organisms. Particularly, the GG motif is not conserved in MoaA in archaea. Therefore, the structures of the C-terminal tail of MoaA/MOCS1A are interesting from medical, structural, and evolutional perspectives.

The catalytic function of the AUX cluster is also incompletely understood. In particular, the electronic coupling between GTP and the AUX cluster is interesting and may have some mechanistic roles. For example, in synthetic Fe[83] and Ni[84] polypyridine complexes, electronic coupling between the metal and ligand induces positive shifts of the reduction potential of the ligand. Therefore, in MoaA, the electronic coupling between GTP and the AUX cluster may further facilitate the aminyl radical reduction and prevent reoxidation of 3′,8-cH$_2$GTP. Also, the conserved R266 and R268 may have roles in the electronic coupling by stabilizing the keto-tautomer, which may explain the catalytic essentiality of these residues and the MoCD disease caused by their mutations. The extent of the electronic coupling between GTP and the AUX cluster and its effects on the aminyl radical reduction are currently under investigation.

Also, there should be a mechanism by which MoaA prevents 3′,8-cH$_2$GTP from being oxidized in the MoaA active site. The calculated reduction potential of the aminyl radical is only 0−25 mV, more positive than that of the experimental reduction potential of the AUX cluster, suggesting that the aminyl radical reduction could be reversible. However, no radical is detectable when the oxidized MoaA is incubated with 3′,8-cH$_2$GTP, methionine, and 5′-dA. Therefore, there must be a mechanism in MoaA that prevents 3′,8-cH$_2$GTP from being reoxidized to aminyl radical. Such a mechanism would be important for Moco biosynthesis considering the limited chemical stability of 3′,8-cH$_2$GTP and the strong affinity of 3′,8-cH$_2$GTP demonstrated by the product inhibition. Two possible mechanisms are conceivable. First is the positive shift of the reduction potential of the aminyl radical through its electronic coupling with the AUX cluster discussed above. Another possibility is the protonation of R17. The reoxidation of 3′,8-cH$_2$GTP requires a proton acceptor. However, under physiological pH, R17 must be protonated and cannot serve as the proton acceptor. Therefore, reoxidation of 3′,8-cH$_2$GTP does not proceed after R17 is reprotonated. Regardless of the mechanisms, the absence of the 3′,8-cH$_2$GTP oxidation in the MoaA active site suggests that the MoaA active site likely provides an inert environment for the chemically labile 3′,8-cH$_2$GTP until it is transferred to MoaC and converted to cPMP.

5. MoaC CATALYTIC MECHANISM

5.1. Overview

The discovery of 3′,8-cH$_2$GTP revealed the catalytic function of MoaC as an enzyme that catalyzes the complex rearrangement reaction between 3′,8-cH$_2$GTP, and cPMP. This finding was surprising as MoaC was not thought to be involved in the rearrangement reaction. Consequently, much less is known about the MoaC catalytic mechanism. However, preliminary structural and mechanistic characterizations have provided insights.

5.2. Structure of MoaC

The structural characterization of MoaC has provided the foundations for studying the MoaC catalytic mechanism. MoaC forms a hexamer composed of a trimer of dimers.[50] The active site is located at the interface of each dimer[11] (Figure 9A,B). In crystals, MoaC adopts two different conformations. WT-MoaC structure was solved with the closed conformation where K51 in loop 3 interacts with D128 (Figure 9B). In this conformation, loop 3 also interacts with the N-terminal loop through backbone amides. The crystal structure of this closed conformation was solved in complex with cPMP, suggesting the ability of this conformation to bind cPMP. The open conformation was found in the K51A-MoaC mutant (Figure 9A), where loop 3 was dissociated from the active site due to the absence of the K51-D128 interaction. Consequently, the N-terminal loop could not interact with loop 3 and was disordered. The crystal structure of K51A-MoaC was solved in complex with 3′,8-cH$_2$GTP, suggesting that the open conformation is still able to bind 3′,8-cH$_2$GTP. The ability of K51A-MoaC to bind 3′,8-cH$_2$GTP was further supported by the observation that this mutant catalyzes the conversion of 3′,8-cH$_2$GTP into intermediate X (see section 5.3). Based on these observations, a conformationally guided catalysis mechanism was proposed (Figure 9C). In this model, the open conformation of MoaC binds 3′,8-cH$_2$GTP and catalyzes its transformation to intermediate X. Subsequently, loop 3 and the N-terminal loop close, allowing MoaC to catalyze the transformation of intermediate X into cPMP.

5.3. Progress in Understanding the MoaC Mechanism

The first chemical evidence for the proposed mechanism was obtained through the characterization of active site variants.[11] Among the six catalytic residues, mutations of K51 or K131 resulted in altered activity of MoaC to transform 3′,8-cH$_2$GTP into another compound (intermediate X). This compound was converted into cPMP when incubated with wt-MoaC, suggesting that it is an on-pathway intermediate or a shunt product that can re-enter the reaction path. While its isolation

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has not been successful due to its limited stability, MS, chemical derivatization, and UV−vis spectroscopy suggested that the intermediate X possesses an acid-labile amino-pyrimidinone moiety and has not established the pyranopterin ring.

MoaC mechanism was also characterized using an uncleavable substrate analogue, 3′,8-cH$_2$GMP[CH$_2$]PP.[10] Unexpectedly, this compound caused a covalent inhibition of MoaC. The resulting MoaC was covalently modified with the mass increase by 503 Da. Since 3′,8-cH$_2$GMP[CH$_2$]PP was converted to an analogue of the intermediate X by K51A- and K131A-MoaC, the observed modification was proposed to be chemically distinct from intermediate X and likely an analogue of a more advanced intermediate. Based on the chemical derivatization study, the modification (compound Y) has not yet established the pyranopterin ring.

Based on these observations, two possible mechanisms using general acid/base catalysis have been proposed (Figure 9D).[10,11] In both of these mechanisms, the reaction is initiated by cleavage of the C8−N9 bond facilitated by electron pushing from N7 and polarization of C6═O through its interaction with E112 and M113 backbone amide protons and the dipole moment of the α-helix. Subsequent hydrolysis of the C8═N7 iminium would reveal an aldehyde intermediate X (mechanism A). Alternatively, a retro-aldol-type reaction followed by C8═N7 iminium hydrolysis would yield another aldehyde intermediate X (mechanism B). Regardless of the mechanism, the resulting intermediate X is less bulky around D128, allowing K51 to be introduced into the active site and interact with D128. The subsequent transformation uses K51 as a general acid/base catalyst. The last step in the catalysis is a concerted formation of the pterin ring and cyclic phosphate ring. The precursor to this final cyclization step would have a nucleophilic ketone moiety that may be susceptible to nucleophilic attack by one of the active site amino acid residues. This mechanism implies that the previously proposed pyranopterin triphosphate (Figure 3A) is unlikely an intermediate of MoaC catalysis or Moco biosynthesis.

While the proposed mechanisms are chemically reasonable, significant ambiguity remains about the mechanism. First, the structure of intermediate X is not known, leaving at least two mechanistic options and potentially more. Also, the mechanistic basis for the covalent inhibition by 3′,8-cH$_2$GMP[CH$_2$]-PP is unknown, raising the possibility of a covalent catalytic mechanism of MoaC. Although no evidence is currently available to support a covalent catalysis, mechanism-based inhibition by other substrate analogues may also be possible. Therefore, further characterization of MoaC inhibition by 3′,8-cH$_2$GMP[CH$_2$]PP is important for understanding the MoaC catalytic mechanism and future development of MoaC inhibitors.

6. TRANSLATING THE MECHANISTIC AND STRUCTURAL UNDERSTANDING TO BIOLOGY AND MEDICINE

6.1. Molecular Basis of Human MoCD Disease

Despite the long history of MoCD, the most common outcome of the disease is the death of the patients in their early childhood. More than 60% of MoCD patients carry mutations in the *MOCS1* gene. Many of such mutations are point mutations in MOCS1A and found in (1) Cys ligands for the 4Fe-4S clusters, (2) three conserved Arg residues in the GTP binding site, and (3) the two conserved Gly residues in the C-terminus.

Studies in bacterial MoaA have so far provided insights into the mechanistic cause of the MOCS1A inactivation through these mutations. The three Arg residues (17, 266, and 268) are strictly conserved and essential for the catalytic functions of bacterial MoaA. As described above, R17 plays a critical role in the transition state stabilization during the 3′,8-cyclization,[14] and therefore its mutation to other amino acids except for Lys completely abolishes the 3′,8-cH$_2$GTP formation.[14,48] The roles of the other two Arg residues, R266 and R268, remain ambiguous. Although their mutations alter the affinity to GTP at varying degree,[48] all the mutants are still capable of binding GTP at physiological concentrations (~0.3−0.5 mM[85]). Still, the catalytic activities of most of the mutants are below the detection limit even with high GTP concentrations (>1 mM). Thus, the mechanism by which their mutations cause complete loss of the MoaA/MOCS1A activity is currently unknown. One possibility is that these residues have a function in maintaining the keto-tautomer of the guanine base for appropriate coordination of the guanine base to the AUX cluster. As described above, the guanine base of GTP is electronically coupled to the AUX cluster. Although the mechanistic significance of this electronic coupling is not yet understood, it is possible that the two Arg residues play key roles in maintaining this electronic coupling and, therefore, are critical for the activity of MoaA.

The function of the two Gly residues in the C-terminus of MoaA/MOCS1A (GG motif) is even less understood. In MOCS1A, mutation of these residues to larger amino acids caused the inactivation of the enzyme based on the gene complementation study in *E. coli*.[86] On the other hand, when *S. aureus* MoaA was tested in vitro, even a mutation of either residue to Ala completely abolished the activity.[76] The potential function of the GG motif in SAM binding was suggested based on peptide complementation assay.[76] In this analysis, a synthetic peptide with an amino acid sequence corresponding to the C-terminal 11 amino acid residues of MoaA successfully rescued the catalytic function of the GG motif mutant of MoaA to the wildtype level activity. With this assay, the potential site of peptide binding was mapped close to the SAM binding site. Also, the GG motif mutation abolished the SAM binding but minimally affected GTP binding. Finally, the analysis of the reported MoaA crystal structures revealed a significant exposure of SAM and GTP to solvent (Figure 7) and suggested that the structure is likely missing a significant portion of the active site. Consequently, the C-terminal tail was proposed to form a part of the active site to provide a seal that isolates the active site from the solvent.[76] In this model, the C-terminal GG motif was proposed to be inserted deeply into the active site, for which the size of the C-terminal amino acid residues is critical. Similar observations were made in other enzymes with conserved Gly residues at the C-terminus, such as ubiquitin and sulfide transfer enzymes,[87] where the C-terminal Gly residues are inserted into the active site of a partner enzyme. In these cases, the C-terminal carboxylate is adenylated for further modification by either ubiquitin or sulfide. In contrast, in MoaA, the GG motif is likely important for the C-terminal tail to be inserted into the spatially confined active site to form the unique environment for the radical catalysis.

While there is currently no established treatment for MoCD, daily injections of cPMP to MoCD patients with mutations in

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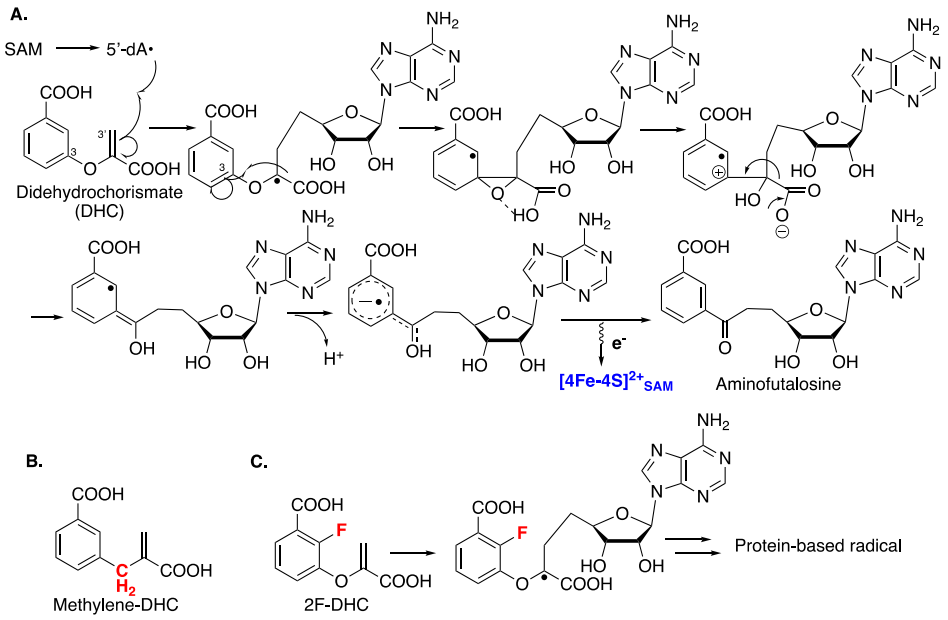

Figure 10. Catalysis and inhibition of MqnE.

the *MOCS1* gene (type A MoCD) have been shown to significantly reduce the severity of the neurological symptoms.[19] Several factors were found to affect the efficacy of the treatment, including the timing of the initiation of the therapy. Since cPMP is irreversibly oxidized by O_2 at physiological pH, the substitution of the cPMP with a chemically more stable surrogate could potentiate the therapeutics. A better understanding of the mechanism and substrate specificity of MoaA and downstream enzymes could allow their use as tools to prepare chemically stable Moco surrogates.

Alternatively, as we understand more about the structures and mechanisms of MoaA/MOCS1A and other Moco biosynthetic enzymes, the chemical rescue of biosynthetic enzymes may become possible. For example, the peptide rescue of the C-terminal GG motif mutations has been demonstrated for bacterial MoaA.[76] Similar activity rescue is likely feasible for human MOCS1A using the peptide with human MOCS1A sequence. While the affinity of 11-mer peptide to MoaA is moderate (150 μM) and the 11-mer peptide with proteinogenic amino acids is unlikely stable in vivo, future structural characterization of this 11-mer peptide and/or intact MoaA may allow the development of more stable and cell-penetrating small molecule surrogate of MoaA active site amino acid residues.

Finally, the recent finding of the ability of *C. elegans* to take up Moco from bait microorganisms is also intriguing in terms of rescuing MoCD.[15] Moco is essential for *C. elegans* and variant strains with mutations in Moco biosynthetic enzymes cannot hatch from eggs. However, such Moco deficient mutants can still grow by feeding with bait microorganisms expressing Moco-dependent enzymes[15] or, more recently, by supplementing Moco-binding proteins.[16] Characterization of this Moco salvage pathway in *C. elegans* may provide hints to the future application to deliver Moco to human MoCD patients.

6.2. Inhibitor of cPMP Biosynthesis

Specific inhibitors of bacterial Moco biosynthesis would be useful to investigate the roles of Moco in virulence and to probe the feasibility of Moco biosynthesis inhibition as novel antivirulent therapy. However, there is currently no specific inhibitor of Moco biosynthesis. Although TCA1 has been reported to be targeting MoeW,[29] no molecular level characterizations have been reported. Also, MoeW is a putative sulfide carrier protein and is potentially involved in other sulfide utilizing pathways such as cysteine and Fe-S cluster biosynthesis, which complicates the interpretation of the outcome of MoeW inhibition. MoaA is the first committed step and likely the rate-limiting step in Moco biosynthesis. Therefore, the inhibition of MoaA likely causes efficient inhibition of Moco biosynthesis and the loss of all the Moco-dependent enzymes.

Since many radical SAM enzymes are potential targets for anti-infectious therapeutics, the development of MoaA inhibitors would also inform the inhibitor development against radical SAM enzymes in general. Inhibitors of enzymes that catalyze radical reactions frequently exhibit mechanism-based inhibition. Such compounds are best known for ribonucleotide reductase, many of which trap radical species in the active site and covalently modify the active site amino acid residues.[88] For radical SAM enzymes, reported inhibitors are limited to those for MqnE. MqnE catalyzes a transformation of dihdehydrochorismate (DHC) to aminofutalosine during the menaquinone biosynthesis. Its catalytic mechanism was proposed to proceed through the addition of 5′-dA• to DHC, followed by a radical rearrangement reaction (Figure 10A). A methylene analogue of DHC (Figure 10B) was shown to reversibly inhibit MqnE with the K_i of 3.1 ± 0.1 μM.[89] The absence of irreversible inhibition was established by restoration of the activity after the removal of the inhibitor. Details of the mechanism of inhibition by this methylene analogue have not been reported. In a separate study, a 2-fluoro-DHC (Figure 10C) was shown to inhibit MqnE with IC_{50} of 35 μM.[90] Intriguingly, in this case, 2-fluoro-DHC served as a substrate and generated a radical on 2-fluoro-DHC but was not converted to 2-fluoroaminofutalosine. Instead, an accumulation of a putative protein-based radical was observed. Based on these observations, 2-fluoro-DHC was proposed to irreversibly inactivate MqnE. Similar mechanism-based inhibition may be

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possible for MoaA. Especially, bacterial MoaA catalyzes a unique shunt pathway and accumulates a 5′-dA-C4′ radical, which is subsequently reductively quenched by a transfer of solvent nonexchangeable proton/hydrogen likely from the protein.[14] Although MOCS1A is not well characterized yet, from an evolutionary perspective, it is possible that the human homologue is better-designed to avoid this shunt pathway. As a result, inhibitors whose design takes advantage of the presence of this shunt pathway could be specific to bacterial enzymes.

Several other approaches for MoaA inhibition are conceivable. First, since GTP is directly coordinated to the AUX cluster, small molecules that bind 4Fe-4S clusters could serve as inhibitors. Such 4Fe-4S cluster-targeting inhibitors have been reported for IspG[91] and IspH[92,93] (Figure 11A), enzymes

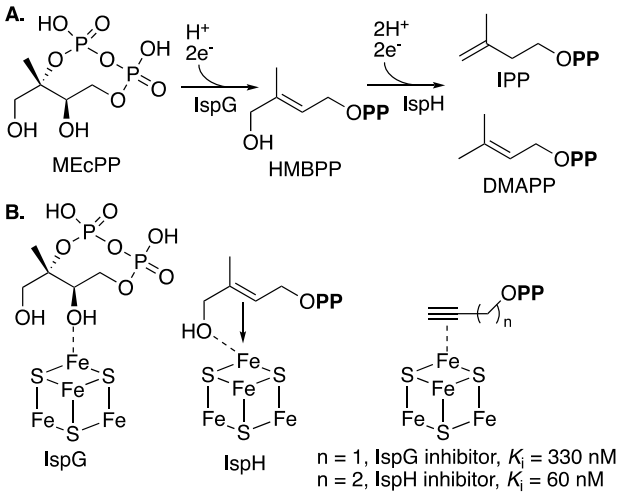

Figure 11. IspG and IspH. (A) Reactions catalyzed by IspG and IspH. (B) Proposed coordination of substrates and inhibitors to the 4Fe-4S clusters of IspG and IspH.

responsible for the nonmevalonate (methylerythritol phosphate, MEP) pathway for isoprenoid biosynthesis. Although these enzymes are not members of radical SAM enzymes, they bind substrates through the vacant coordination site of their 4Fe-4S clusters (Figure 11B). Potent inhibition (K_i as low as 60 nM) of these enzymes was observed with molecules with stronger Fe coordination capability compared to the substrates (Figure 11B). Second, although unprecedented to our knowledge in enzymes that catalyze radical reactions, transition state analogues of MoaA catalysis could exhibit potent inhibition. The mechanistic characterization of MoaA suggested that MoaA accelerates the addition of GTP C3′• to C8 by stabilizing the transition state.[14] Since the transition state structure could be different between bacterial vs human enzymes, transition state analogue could also achieve the selectivity between bacterial MoaA vs human MOCS1A. Finally, since the C-terminal tail of MoaA likely reversibly interacts with the active site, it may be possible to inhibit MoaA with a conformationally rigid C-terminal tail mimic that minimizes the entropy loss upon binding. The structural determination of the intact MoaA active site may allow the design of the conjugate between the C-terminal tail and a GTP analogue. Since the C-terminal tail amino acid sequence is distinct between bacterial MoaA and human MOCS1A, selective inhibition of bacterial MoaA may be possible by targeting the C-terminal tail binding site.

Alternatively, for antibiotic development, dual targeting of Moco and 4Fe-4S cluster biosynthesis may be more effective as a 4Fe-4S cluster is essential for the function of MoaA and many Moco-dependent enzymes.[94] In *E. coli*, Fe-S cluster insertion to MoaA is mediated by the Isc system and an A-type carrier protein ErpA under oxygen-limiting condition.[95] *E. coli* grown under iron limiting conditions show significantly reduced intracellular Moco production due to the dysfunctional [4Fe-4S] assembly and failure to produce functional MoaA and the downregulation of L-cysteine desulfurase essential for MPT synthase[96] (Figure 1). Therefore, specific inhibition of Fe-S cluster biosynthesis or sulfur trafficking in pathogenic bacteria may provide potent growth inhibition by pleiotropic loss of both FeS cluster and Moco.

Overall, the functionally rich MoaA active site may allow inhibitor development by one of the above-mentioned approaches or their combinations. Further structural and mechanistic studies on MoaA will facilitate such development.

7. CONCLUSION

The elucidation of the functions of MoaA and MoaC revealed new opportunities in studying the catalytic mechanisms and chemical biology/medicinal chemistry of these enzymes to address outstanding questions in the field of Moco biosynthesis or even broader human health-related problems. Especially, although Moco has been studied since the 1960s, its role in bacterial pathogenesis has come to light only in the past decade. Considering the significance of anaerobic metabolism and respiration during virulence of pathogenic bacteria, a better understanding of the role of Moco and its biosynthesis in these processes is important for our eventual success in combatting difficult-to-treat bacterial infectious diseases.

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ABBREVIATIONS

Moco, molybdenum cofactor; 3′,8-cH$_2$GTP, 3′,8-cyclo-7,8-dihydroguanosine 5′-triphosphate; cPMP, cyclic pyranopterin monophosphate; MPT, molybdopterin; GTP, guanosine 5′-triphosphate; SAM, *S*-adenosyl-L-methionine; MoCD, molybdenum cofactor deficiency

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