Mechanistic Characterization of Cyclic Pyranopterin Monophosphate Formation in Molybdenum Cofactor Biosynthesis

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in the Graduate School of Duke University

2014
ABSTRACT

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Abstract

The molybdenum cofactor (Moco) is an essential enzyme cofactor found in all kingdoms of life. Moco plays central roles in many vital biological processes, and must be biosynthesized de novo. During its biosynthesis, the characteristic pyranopterin ring of Moco is constructed by a complex rearrangement of guanosine 5′-triphosphate (GTP) into cyclic pyranopterin (cPMP) through the action of two enzymes, MoaA and MoaC. However, the mechanisms and the functions of the two enzymes are under significant debate. To elucidate their physiological roles, I took a multidisciplinary approach to functionally characterize MoaA and MoaC in vivo and in vitro. In this dissertation, I report the first isolation and characterization of the physiological MoaC substrate, 3′,8-cyclo-7,8-dihydro-guanosine 5′-triphosphate (3′,8-cH₂GTP). I also report the first X-ray crystal structures of MoaC in complex with this highly air sensitive substrate, and its product cPMP. These studies, combined with in vitro experiments using substrate analogs, catalytically impaired mutants, and synthetic peptides, have enabled me to delineate the functions of the Moco biosynthetic enzymes, MoaA and MoaC, and proposed mechanistic models for their roles in the formation of cPMP.
Dedication

To the strongest,
most inspirational
person I know:
    Me
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List of Commonly Used Abbreviations

3ˊ,8-chGTP – 3ˊ,8-cyclo-7,8-dihydro-guanosine 5ˊ-triphosphate
5ˊ-dA – 5ˊ-deoxyadenosine
5ˊ-dA• – 5ˊ-deoxyadenosyl radical
CmdZ – Compound Z
cPMP – Cyclic pyranopterin monophosphate
DMPT – Dimethylpterin
DTH – Dithionite
DTT – Dithiothreitol
EPR – Electron paramagnetic resonance
FPLC – Fast protein liquid chromatography
GTP – Guanosine 5ˊ-triphosphate
GMPcPP – Guanosine-5’-[α,β]-methylene]triphosphate
HPLC – High performance liquid chromatography
ITC – Isothermal titration calorimetry
LC-MS – Liquid chromatography-mass spectrometry
Mo – Molybdenum
MoaA – Moco biosynthetic enzyme A in bacteria
MoaC – Moco biosynthetic enzyme C in bacteria
MocD – Molybdenum cofactor deficiency
Moco – Molybdenum cofactor
MOCS1A – Moco biosynthesis enzyme A (human homolog to bacterial MoaA)
MOCS1B – Moco biosynthesis enzyme A (human homolog to bacterial MoaC)
MPT – Molybdopterin
NMR – Nuclear magnetic resonance
PCR – Polymerase chain reaction
PPP - Triphosphate
SAM – S-adenosyl-L-methionine
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1. Introduction

1.1. Overview

The molybdenum cofactor (Moco, Figure 1a, 4) is a metal-containing prosthetic group common to nearly all molybdoenzymes and ubiquitous to all kingdoms of life [1-6]. Moco-dependent enzymes play central roles in many biologically important processes such as purine and sulfur catabolism in mammals, anaerobic respiration in bacteria, and nitrate assimilation in plants [2]. In humans, Moco deficiency results in the pleiotropic loss of all molybdenum enzyme activities, causing neurological abnormalities and early childhood death [7, 8]. Unlike many other cofactors, Moco cannot be taken up as a nutrient, and thus requires de novo biosynthesis (Figure 1a). Moco biosynthesis is thought to be conserved among all organisms [5, 9] and initiated by the conversion of the nucleotide, guanosine triphosphate (GTP, Figure 1a, 1), into cyclic pyranopterin monophosphate (cPMP, Figure 1a, 2) [10-12]. cPMP is then converted to Moco by the introduction of two sulfur atoms (MPT, Figure 1a, 3) [13-15] and a molybdate (Figure 1b, 5) [16-19].
(a) Moco biosynthetic pathway in bacteria and humans. Molybdenum cofactor (Moco) biosynthesis occurs in three steps: (i) the formation of cyclic pyranopterin monophosphate (cPMP) from the nucleotide, guanosine triphosphate (GTP), (ii) the conversion of cPMP into molybdopterin (MPT), (iii) the insertion of molybdate into MPT to form Moco. The human enzymes are indicated in parenthesis. The symbols on GTP and cPMP indicate the source of the carbon and nitrogen atoms in cPMP as determined by isotope labeling studies[10, 20]. P designates a phosphate group. (b) Illustration of Moco biosynthesis in a bacterial cell. Molybdate is transported into the cell through the high-affinity molybdate transporter, ModABC. Once in the cell, molybdate combines with molybdopterin to form the molybdenum cofactor. Moco is subsequently inserted into a diverse group of Moco-dependent molybdoenzymes.
1.2. The Molybdenum Cofactor

1.2.1. Discovery of Molybdenum and its Cofactor

The transitional element molybdenum was first discovered in 1778 by Swedish chemist, Carl Wilhelm Scheele [21]. Three years later, fellow Swedish chemist, Peter Jacob Hjelm, successfully isolated molybdenum from the ore, molybdenite, naming it after the Ancient Greek word, Μόλυβδος (molybdos), meaning lead or lead-like [22]. In the 1930s, the biological relevance of molybdenum to living organisms was first demonstrated to be essential for plant growth and nitrogen fixation in bacteria [23, 24], and several decades later, the importance of molybdenum to human health was first proposed [25-27].

In biological systems, molybdenum is bioavailable as the oxyanion molybdate (MoO$_4^{2-}$). Molybdate enters both prokaryotic and eukaryotic cells primarily through the well-conserved and high affinity ATP-binding cassette, ModABC (Figure 1b) [28, 29]. Once imported into the cell, molybdate is incorporated into a metal cofactor by a series of biosynthetic enzymes. While molybdate is redox-active under physiological conditions, it requires a prosthetic group to orient and insert itself in molybdoenzymes [30]. Once in an enzyme, the Mo-coordinated cofactor catalyzes both one and two-electron reductions and oxidations over a wide range of redox potentials by utilizing molybdenum’s multiple oxidation states (+4, +5, and +6). It is this versatile redox chemistry that enables the wide variety of chemical transformations by molybdenum.
While now recognized as an essential component to many redox enzymes, it was not until the 1960s that the first evidence for the existence of a molybdenum cofactor came to light. In the course of characterizing genetic mutations affecting the activity of two enzymes in *Aspergillus nidulans*, nitrate reductase (NR) and xanthine dehydrogenase (XDH), Cove and Pateman discovered pleiotropic mutants that produced inactive forms for both of these seemingly independent enzymes [31, 32]. The group hypothesized that these activity-impaired *A. nidulans* mutants carried mutations in genes involved in the biosynthesis of a previously undiscovered cofactor common to both XDH and NR. Similar mutant strains were subsequently identified in a diverse range of organisms [33-38]. Eventually, the identity of these genes required for Moco biosynthesis was revealed through the extensive phenotypic screening of *E. coli* mutants [33, 39, 40].

1.2.2. The Structure of the Molybdenum Cofactor

The first chemical characterization of the molybdenum cofactor came through the seminal work of Rajagopalan and coworkers (Figure 2) [41, 42]. Unbound Moco, like many of its precursor molecules, is extremely unstable outside of the controlled environments found inside molybdoenzymes. As a result, Moco is difficult to study in its active form [41, 43, 44]. To overcome this issue, Rajagopalan and coworkers derivatized freshly isolated Moco to a combination of stable degradation products, which effectively allowed them to probe the structure of the labile cofactor [43]. Generated by oxidizing Moco with either acidic iodine or air, two distinct and stable
fluorescent derivatives were obtained, purified, and then subsequently structurally characterized. These oxidized derivatives (known as Form A and B, Figure 2, 7 & 8 respectively) suggested that the cofactor consisted of a unique pterin ring modified with a phosphorylated dihydroxybutyl sidechain containing a \textit{cis}-dithiolene bond. Actual evidence of the two vicinal sulfurs came from careful comparison of the structurally similar Form B and the pterin molecule urothione (Figure 2, 10) that also contains two sulfur atoms. Urothione, a previously identified metabolite excreted in urine [45], is intriguingly not found in patients suffering from Moco deficiency, and thus was hypothesized to be metabolically linked to Moco biosynthesis[41]. Since urothione could readily be converted to the dephosphorylated form of Form B by desulfuration of the –SCH$_3$ group, it was suggested that Form A and Form B were produced by the loss of one or both of these sulfurs. Additional structural support for the likely labile dithiolene moiety was obtained by iodoacetamide-treatment of Moco to form a stable di-(carboxamidomethyl)-derivative (camMPT, Figure 2, 9) [46]. This observed derivative supported the hypothesis that Moco was a previously uncharacterized alkylated pterin that coordinates molybdate using a \textit{cis}-dithiolene group at the C8 and C3’ (numbering based on guanosine, Figure 1a).

Altogether, this novel pterin structure in its metal-free form became known as Molybdopterin (MPT, Figure 2, 3), despite the lack of molybdenum. Several years after the original characterization by Rajagopalan, the first crystal structures of
molybdoenzymes [47-49] corroborated the proposed structures of MPT and Moco. The only exception from the otherwise valid model was that the proposed dihydroxybutyl side chain actually forms a pyran ring by intramolecular cyclization. This tricyclic pyranopterin-form of Moco is considered the complete and simplest form of the molybdenum cofactor [5].
Figure 2: Structural Determination of Moco and its Precursors

Summary of the structural characterization of Moco and its precursor molecules completed by Rajagopalan and colleagues [41, 42]. Shown are the structures of Moco and its precursors: GTP, cPMP, MPT. cPMP can exist in solution as a ketone form (cPMP, 2) or as hydrated form (cPMP, 2b). Illustrated here is cPMP and air-oxidized, fluorescent product, Compound Z. MPT and Moco can both be derivatized to Form A with acidic iodine treatment or Form B with air-oxidation. MPT can be additionally derivatized to a stable di-(carboxamidomethyl)-MPT, known as camMPT. Urothione, a structurally similar molecule to Form B, is the natural catabolic degradation of Moco. Main biosynthetic pathway for Moco is indicated with black arrows, while chemical derivatization and catabolic degradation is indicated with red arrows. Figure adapted from S. Leinkuhler et al. [5].
1.2.3. Molybdenum Cofactor-containing Enzymes

From the aforementioned structural studies and additional chemical characterization [50, 51], it is now known that Moco is commonly found in three distinct forms in molybdoenzymes (Figure 3) [4, 6]. Historically, molybdoenzymes have been categorized into discrete families based on these modifications to Moco. These include: the sulfite oxidase (SO) family, the xanthine oxidase (XDH) family, and the DMSO reductase (DMSOR) family.

The SO family of molybdoenzymes, while the least populated of the three families in terms of identified enzymes, are conserved across all kingdoms. In addition, they are essential for life in higher organisms as sulfite oxidase is critical for healthy neuronal development [52]. Enzymes in this family utilize a single Moco pterin molecule that consists of a MPT-Mo$^{VI}$O$_2$ core with a cysteine covalently coordinated between the molybdoprotein and Mo (Figure 3, 11) [30]. The catalysis generally involves a sulfite oxidation coupled to a molybdenum reduction [53]. For mammalian sulfite oxidases, molybdenum reduction is followed by a two-electron transfer step through a molybdoenzyme-bound cytochrome-\textit{b}$_5$-type heme to cytochrome \textit{c}, a hemeprotein involved in the electron transport chain. Sulfite oxidase-like enzymes found in plants are localized in the peroxisomes and lack the cytochrome-\textit{b}$_5$ heme domain, and instead form a homodimer of two Moco-binding subunits [6, 54]. The SO family of molybdoenzymes found in bacteria on the other hand form cytoplasmic heterodimers from a Moco-
binding subunit and a cytochrome c-like subunit, all together bypassing the requirement of a cytochrome-\textit{b5} heme in sulfite oxidation.

The XDH family of molybdoenzymes catalyzes the two-electron transfer hydroxylation of carbon centers in various naturally occurring aldehydes and purines [55]. For example, the Moco-dependent xanthine oxidase catalyzes the medical significant oxidation of hypoxanthine and xanthine to uric acid during purine catabolism. The XDH family of enzymes utilize a similar single MPT-Mo\textsuperscript{V}O(OH) core as found in SO enzymes, but has a terminal sulfido ligand bound to Mo instead of the cysteine. A variation of this XDH family sulfurated Moco has been found in the \textit{E. coli} periplasmic aldehyde oxidoreductase, PaoABC, harboring a cytosine monophosphate attached to the single Moco phosphate moiety (Sulfurated MCD, Figure 3, 12) [56]. By comparison to the SO family, XDH enzymes are molybdo-flavoenzymes that form hetero-oligomeric complexes with Moco-containing subunits and [2Fe-2S] cluster- and FAD-binding subunits. Utilizing the sulfurated Moco, XDH and its homologs oxidize their substrates by reducing molybdenum and shuttling the electrons through the [2Fe-2S] cluster and FAD to molecular oxygen derived from water [55].

The largest group of molybdoenzymes are the DMSOR family, which vary significantly in their structure and function, but all contain a Moco derivative that has two equivalents of the pterin moieties coordinating the Mo metal [4]. Each of the two pterin molecules also contain a guanosine monophosphate attached to the Moco
phosphate group in an analogous manner to that of the cytosine monophosphate in the XDH family. The molybdenum coordination at the core of this cofactor is completed by either a terminal oxo or sulfido ligand, and a sixth amino acid derived or hydroxyl ligand that varies from enzyme to enzyme within the family [57]. All together, this variation on the Moco for the DMSOR family is known as the Mo-bis-MPT guanine dinucleotide cofactor (Mo-bis-MGD, Figure 3, 13). DMSOR enzymes are typically membrane-associated or periplasmic complexes consisting of subunits harboring not only the Mo-bis-MGD cofactor, but also multiple [3Fe-4S] or [4Fe-4S] clusters, and cytochrome-\textit{b5}-type hemes [58]. A majority of the enzymes belonging to the DMSOR family, such as DMSO reductases, formate dehydrogenases, biotin sulfoxide reductases, and nitrate reductases are only found in prokaryotes, and are generally involved in anaerobic respiration by acting as terminal reductases, although many have also been implicated in several other versatile redox reactions [4]. Due to the medical significance of anaerobic respiration in chronic and persistent bacterial infections, the bacterial-specific maturation and insertion of Mo-bis-MGD into the DMSOR enzymes has been potent target for therapeutics and drug discovery [59].
Overview of the three families of molybdoenzymes and their uniquely modified molybdenum cofactors. These families are: sulfite oxidase (SO), xanthine oxidase (XDH), and DMSO reductase (DMSOR) families. The SO family of molybdoenzymes utilizes a di-oxo form of Moco with protein-based cysteine ligand. The XDH family contains a form of Moco sulfurated by a reversible L-cysteine desulfurase and is further modified with cytosine dinucleotide by MocA (Sulfurated MCD). The DMSOR family uses two ligated forms of Moco with guanosine dinucleotide modifications catalyzed by MobA (Mo-bis-MGD). The single Mo in Mo-bis-MGD is also ligated by either an oxo or sulfido ligand, and a sixth ligand, which is a protein-derived amino acid or hydroxyl ligand.
1.3. Molybdenum Cofactor Biosynthesis

The early understanding of Moco biosynthesis was achieved through the careful genetic and phenotypic screening of *E. coli* strains with mutations along the proposed biosynthetic pathway [33, 39, 40]. Over the last several decades, the use of additional biochemical and structural approaches has allowed for the identification of at least 16 *E. coli* genes involved in Moco biosynthesis (Figure 4) [5]. In *E. coli*, these genes are located across six mo (molybdenum) loci: *moa, mob, moc, mod, moe*, and *mog*. The loci *moa, moc, moe*, and *mog* contain the genes for enzymes directly involved in Moco biosynthesis, and are highly conserved across various Moco-dependent organisms, despite some variability in gene organization between species [60]. The remaining loci are responsible for producing the molybdate transporter (ModABC), Mo-dependent transcriptional regulation, molybdoenzymes-specific modifications, or are pleiotropically linked to Moco biosynthesis but with unidentified functions.

Molybdenum cofactor biosynthesis in bacteria (Figure 5a) can be divided into three primary steps: (i) the formation of cyclic pyranopterin monophosphate (cPMP) from the nucleotide guanosine triphosphate (GTP) [10-12], (ii) the conversion of cPMP into MPT [13-15], (iii) the insertion of molybdate into MPT to form Moco [16-19]. Additional modifications to Moco can occur following this last step of the biosynthesis to create the previously described molybdoenzymes-specific Moco-derivatives like Mo-bis-MGD.
Organization of gene loci required for Moco biosynthesis in *E. coli*. The 16 identified Moco biosynthetic genes are located across six loci: *moa, mob, moc, mod, moe,* and *mog*. As illustrated above, the loci *moa, moc, moe,* and *mog* contain the genes for enzymes directly involved in Moco biosynthesis. The remaining genes within these loci are responsible for producing the molybdate transporter (ModABC), transcriptional regulation (*modE*), or are pleiotropically linked to Moco biosynthesis but with unidentified functions (*moaB, mobB,* and *modF*). Genes above are drawn approximately to scale and are colored by the identified function. Asterisks indicate promoter regions for each operon. Figure adapted from C. Iobbi-Nivol et al. [4].

**1.3.1. Conversion of GTP into cPMP**

Moco biosynthesis is initiated with the complex chemical conversion of GTP to cPMP, an alkylated pterin molecule with a cyclic phosphate moiety. This reaction is the rate-limiting step of Moco biosynthesis and historically considered to be the first stable intermediate of the pathway [61, 62]. Ubiquitously found in nature, GTP serves as a common starting substrate for several pterin and flavin biosynthetic pathways,
including but not limited to folate, riboflavin, and biopterin [5, 63-65]. Using isotopically labeled GTP, Rajagopalan and colleagues demonstrated that not only is cPMP formed from a guanosine-based starting material, but is also mechanistically intriguing in that the C8 of guanine is inserted between the C2' and C3' of ribose (Figure 1a) [10]. This is in sharp contrast to other pterin biosynthetic pathways, where the C-8 of guanine is hydrolyzed by GTP cyclohydrolases (Figure 5a) [64, 65]. The conversion of GTP into cPMP requires two enzymes, MoaA and MoaC. However, thus far no consensus has been established about the mechanism of the reaction, or even about the individual functions of MoaA and MoaC.
Figure 5: Pterin and Flavin Biosynthetic Pathways

(a) The reactions catalyzed by GTP cyclohydrolases (GTPCH) in the biosynthesis of pterins and flavins. Unlike the synthesis of cPMP, the typical biosynthesis of pterins and flavins results in the loss of C8 from GTP as formate. C8 is indicated with red circle, and P represents phosphates. (b) Common pterins and flavins derived from GTP. Biopterins are act as cofactors for aromatic amino acid hydroxylases, while methanopterin is a cofactor used by methanogenic organisms. Similarly, flavin adenine dinucleotide (FAD) is a critical redox cofactor. Folate and riboflavin are both in the vitamin B family, and are essential for numerous bodily functions.

1.3.1.1. MoaA, A Radical SAM Enzyme

Bioinformatic analysis suggests that MoaA belongs to the newly identified radical SAM (S-adenosyl-L-methionine, Figure 6a, 14) superfamily, a group that includes enzymes such as biotin synthase, pyruvate formate-lyase activating enzyme, and lysine aminomutase [66]. Enzymes in this superfamily catalyze the reductive
cleavage of SAM using an oxygen-labile [4Fe-4S] cluster as a reductant, and transiently generates a 5’-deoxyadenosyl radical (5’-dA•, Figure 6a, 15). The 5’-dA• then abstracts an H-atom from either the substrate to directly initiate a radical reaction, or a glycyl residue to activate the primary catalytic enzyme [67].

A defining feature in radical SAM enzymes is the conserved CxxxCxzC motif (x = Any residue, z = tyrosine, phenylalanine, histidine, or tryptophan), which coordinates the iron sulfur cluster involved in the reductive cleavage SAM (Figure 6b) [68]. Ligated by the 3 cysteines, the oxygen sensitive cluster is reduced to [4Fe-4S]1+ using either a physiological or exogenous reductant and then subsequently cleaves a cluster-bound SAM molecule to generate the 5’-dA•. In the case of MoaA, X-ray crystal structures of Staphylococcus aureus MoaA [12] revealed SAM as the fourth ligand to a 3 cysteine-coordinated [4Fe-4S] cluster, in a fashion similar to other radical SAM enzymes [68-70]. Similarly, crystal structures show the core of MoaA consisting of the same “partial” TIM barrel fold found canonically in radical SAM enzymes. This fold is characterized by an alternating motif of six all-parallel β strands and six α helices (Figure 6c). The “partial” aspect of the fold found in radical SAM enzymes refers to the fact that a complete TIM barrel fold contains an additional two β/α units. For MoaA, the missing β/α units are replaced with an additional C-terminal [4Fe-4S] cluster, which effectively encloses and completes the active site of the enzyme [12]. This C-terminal [4Fe-4S] cluster was shown to bind various purine nucleoside 5’-triphosphates including GTP in crystal structures.
(Figure 6d) [71] as well as in solution based on electron-nuclear double resonance (ENDOR) spectroscopy [72]. These observations, together with the reported binding constant (0.29 mM) [71] led to the proposal that GTP was a substrate of MoaA. As many radical SAM enzymes are known to catalyze complex rearrangement reactions [67, 73], MoaA has also been considered to catalyze the majority, if not all, of the complex rearrangement of GTP to form the pyranopterin ring of cPMP [5, 9, 74, 75].
Figure 6: MoaA, A Radical SAM Enzyme

(a) Radical SAM enzymes reductively cleave S-adenosyl-L-methionine (SAM) using a reduced [4Fe-4S] cluster and generate a 5′-deoxyadenosyl radical (5′-dA•). The 5′-dA• then abstracts a H-atom from either the substrate to directly initiate a radical reaction, forming 5′-deoxyadenosine (5′-dA) in the process. (b) Sequence homology of several identified bacterial radical SAM enzymes. Included *S. aureus* oxygen-independent coproporphyrinogen III oxidase (HemN), *E. coli* lysine aminomutase (LAM), *S. aureus* pyruvate formate-lyase activating enzyme (PFL-AE), *E. coli* ribosomal protein S12 methylthiotransferase (RimO), *S. aureus* molybdenum cofactor biosynthetic protein A (MoaA), *C. glomerans* [Fe-Fe] hydrogenase maturase protein (HydE), *E. coli* biotin synthase (BioB). Colored residues illustrate the radical SAM superfamily conserved Fe-S cluster-SAM, CxxxCxxC (x = Any residue, z = tyrosine, phenylalanine, histidine, or tryptophan). Amino acid residue numbering based on MoaA. (c) Structure of *S. aureus* MoaA monomer, highlighting the partial TIM barrel motif. β strands are in purple and α helices are in blue. (d) Model of *S. aureus* MoaA active site created by overlaying the structures of MoaA in complex with GTP, and MoaA in complex with SAM [12].
1.3.1.2. MoaC, the Underappreciated Partner in cPMP Formation

MoaC has historically been disregarded as having any significant role in the formation of cPMP. Having no significant amino acid sequence or structural similarities to functionally characterized proteins, the contribution of MoaC has remained a mystery. Previously, structures of MoaC from four different organisms have been reported [76-79]. In the absence of an identified substrate, a majority of these structures were solved as the apo-form of MoaC. Nevertheless, a putative ligand binding site was proposed based on the conservation of amino acid residues and their relative positions in a crystal structure [78]. Eventually, a structure of *Thermus thermophilus* MoaC bound to the non-physiological substrate GTP was obtained, supporting the proposed ligand-binding site [77]. In combination with this structure, isothermal titration calorimetry experiments completed by the same group using mono-, di-, and trinucleotides suggested that nucleotide triphosphate may bind to the putative ligand-binding site. While these results suggested that MoaC may be involved in the cleavage of a pyrophosphate from a MoaA product to form the cPMP cyclic phosphate, the lack of any substantial enzymological characterization of these two enzymes and their potential intermediates left considerable uncertainty in the mechanism of cPMP formation [5].

1.3.1.3. cPMP Formation in Eukaryotes

In humans, the *MOCS1* (molybdenum cofactor synthesis) locus encodes for the homologs of MoaA and MoaC, MOCS1A and MOCS1B, respectively [80], while in
plants, these homologs are Cnx2 and Cnx3 (cofactor for nitrate reductase and xanthine dehydrogenase) [52]. In the case of humans, the MOCS1A and MOCS1B genes are located together in an atypical bicistronic transcript [81]. Through alternative splicing, the stop codon that typically separates the two genes can be bypassed, ultimately allowing for the generation at least 2 identified MOCS1A and MOCS1B fused protein variants (Figure 7) [82]. Unlike MOCS1A, there is no evidence that MOCS1B is expressed naturally as anything but a splice-type fusion with MOCS1A [83]. MOCS1B has been recombinantly expressed, though, and shown to be catalytically active in vitro without the MOCS1A domain [61]. Furthermore, these human homologs to MoaA and MoaC have been shown in vivo to successfully complement ΔmoaA and ΔmoaC E. coli mutants, suggesting the mechanism of cPMP formation is likely conserved in all organisms [83].

Unlike bacterial cPMP formation, which occurs in the cytoplasm, it has been suggested that this step of Moco biosynthesis occurs in the mitochondria in higher organisms [83, 84]. With all of the identified variants of MOCS1A and MOCS1B containing a mitochondrial targeting sequencing, cPMP is suggested to be synthesized and transported into the cytosol using the mitochondrial ABC transporter, ATM3. In a study completed by Bittner and colleagues, Δatm3 A. thaliana mutants accumulated considerable amounts of cPMP in the mitochondria, and exhibited a global decrease in overall levels of Moco [84]. Furthermore, antibodies against Cnx2 and Cnx3 showed that
the proteins were localized in the mitochondrial matrix. The remaining steps of Moco biosynthesis in eukaryotes are localized to the cytoplasm like in prokaryotes.

**Figure 7: Alternative Splicing Variants in Human Homologs of MoaA & MoaC**

The genes for human homologs to the bacterial MoaA and MoaC, MOCS1A and MOCS1B, respectively, are located together in a bicistronic transcript. Through alternative splicing, the stop codon that typically separates the two genes can be bypassed, ultimately allowing for the generation at least 3 identified MOCS1A and MOCS1B fused protein variants. As there is no evidence that MOCS1B is naturally expressed in humans, its required catalytic activity likely derives from the Type II and III fusion proteins with MOCS1A.

### 1.3.2. Maturation of cPMP to Moco

In the later steps of Moco biosynthesis, cPMP is converted first to MPT, and then subsequently forms Moco through the insertion of molybdate (Figure 1a). Compared to the formation of cPMP, these latter steps of Moco biosynthesis are considerably better.
understood given the extensive characterization of the involved biosynthetic enzymes and structural similarities of various intermediates [4, 5]. For example, cPMP is structurally identical to MPT, except it lacks the dithiolene moiety need to ligate the molybdenum-containing oxyanion, molybdate. This dithiolene group is incorporated into cPMP through the insertion of the two sulfur atoms at C8 and C3 of cPMP, a step catalyzed by the enzyme complex, MPT synthase [85, 86]. This complex is a heterodimer of two identical MoaD and MoaE subunits [87, 88], and completes the sulfur insertions through the formation of thiocarboxylate intermediates on the C-terminus of MoaD, a process mediated by the adenylylase, MoeB [62].

In bacteria, molybdate is subsequently inserted into MPT at the dithiolene moiety using two enzymes, MogA and MoeA. MogA catalyzes the ATP-dependent formation of an activated MPT-adenylate intermediate [17, 89], while MoeA acts a high-affinity chelator of molybdate, and mediates the ligation into the pterin scaffold [90]. For eukaryotes, MogA and MoeA exist as a fused two-domain protein: Cnx1 in plants [91], or the synaptic anchoring protein, gephyrin, in mammals [92].

As described above, further modifications to Moco can occur after biosynthesis but prior to insertion into molybdoenzymes. Some of the genes contained within the mo loci encode for proteins responsible for these modifications. MobA for example, catalyzes the formation of Mo-bis-MGD (Figure 3, 13) found in the DMSO reductase family of molybdoenzymes through a two-step process requiring two molecules of GTP
and two molecules of Moco [93-95]. Moco can also be further modified to the sulfurated MCD (Figure 3, 12) found in the XDH family of molybdoenzymes through the combined action of the CTP-specific paralog of MobA, MocA [96], and L-cysteine desulfurases [97].

1.3.3. Regulation of Moco Biosynthesis

A majority of the genes required for the early steps of Moco biosynthesis in E. coli are clustered with the moa operon (moaABCDE), and serve as the main target for transcriptional and translational regulation for the pathway [5, 98]. Regulation of the moa operon is controlled by three distinct upstream elements that sense the cellular levels of molybdate, Moco, and oxygen, and thus ensure the Moco is both required and has the components necessary for de novo synthesis.

At elevated levels of molybdate, the Mo-dependent transcriptional regulator protein ModE binds molybdate forming a complex that in turn interacts with the moa promoter region and enhances transcription of the operon. Additionally, the ModE-molybdate complex has been found to negatively regulate the transcription of the mod operon (modABCD), which encodes for the high-affinity molybdate transporter [99]. This prevents the unnecessary transport of molybdate into the cell if the demands are limited. In contrast to molybdate, elevated levels of Moco in the cell have been shown to down-regulate the moa operon expression, as it implies the cellular requirement for additional Moco is small [98, 100]. Located between the moa promoter region and moa operon is a highly conserved Moco-binding RNA riboswitch, which tightly controls the gene
expression. The Moco-sensing riboswitch binds free Moco when present at higher concentrations and prevents the translation of proteins encoded by the moaABCDE genes. This type of mechanism allows for a rapid and dynamic control of Moco levels within the cell.

As Moco in bacteria is primarily required for anaerobic respiration, the transcription of the moa operon is also down-regulated by the presence of oxygen in the surrounding environment. This process is facilitated by the fumarate and nitrate reduction (FNR) regulatory protein, which contains an oxygen-sensitive [4Fe-4S] cluster [98, 101, 102]. At low levels of oxygen, FNR and its intact [4Fe-4S] cluster bind upstream of the moa promoter region and enhance expression. However, as ambient levels of oxygen in the environment are increased, the [4Fe-4S] clusters in FNR are oxidized, which in turn disorders the transcriptional regulator leaving it unable to interact with the moa operon. This type of oxygen-sensing regulation by FNR is essential not only for Moco biosynthesis, but is a well-conserved process involved in the expression of a large number of anaerobic respiration-related pathways [101].
The Moco biosynthetic pathway is primarily transcriptional and translational regulation at the \textit{moa} operon. Regulation is control by three distinct upstream elements that sense the cellular levels of molybdate, Moco, and oxygen. ModE, the Mo-dependent transcriptional regulator, binds molybdate at high concentrations and up-regulates the pathway. The FNR regulatory protein senses ambient levels of oxygen, and shuts down Moco production under aerobic conditions. Similarly, Moco-specific riboswitch found immediately upstream of the \textit{moa} operon binds excess cellular Moco and down-regulates the pathway. Genes and regulatory elements above are drawn approximately to scale and are colored by the identified function. Figure adapted from S. Leimkuhler et al. [5].

\section*{1.4. Significance of the Molybdenum Cofactor}

\subsection*{1.4.1. Global Significance}

The molybdenum cofactor is utilized by nearly every organism across all kingdoms of life [5, 9]. This ancient and universal cofactor is biosynthesized \textit{de novo} in a highly conserved pathway with homologs in everything from archaea to humans. Exploiting the versatile redox nature of molybdenum, Moco has been demonstrated to play a central role in various biological processes including detoxification pathways in humans [7], causation of bacterial infections [103], and the regulation of global nitrogen-,
sulfur-, and carbon-cycles[104]. The variety of diverse chemical reactions characterized by the greater than 50 identified Moco-containing enzymes has made Moco and its essential biosynthesis a globally significant and vital metabolic pathway.

1.4.2. Medical Significance of the Molybdenum Cofactor

1.4.2.1. Molybdenum Cofactor Deficiency in Humans

Human Moco deficiency (MoCD) is the fatal autosomal recessive disease associated with an inability to produce Moco in the body [6, 105]. Genetic mutations in the human Moco biosynthetic enzymes are responsible for this metabolic disorder and, in turn, the combined loss of activities of all Moco-dependent enzymes. Chiefly, the pleotropic effects of MoCD on the essential sulfite oxidase, xanthine oxidase, and aldehyde oxidase enzymes contribute to nearly all of the symptoms associated with the disease [105]. The loss of activity of sulfite oxidase in particular leads to progressive neurological damage, seizures, and the eventually death of the infant after only a few weeks [106]. Sulfite oxidase, localized in the intermembrane space of the mitochondria, oxidizes the cytotoxic catabolite sulfite into sulfate (Figure 9) [6]. Without Moco to facilitate this reaction, sulfite accumulates in the brain, where it has been suggest to both inhibit ATP synthesis [107], and reduce cysteine to S-sulfocysteine, an unnatural agonist to the neurologically significant GABA receptor [108]. Schwarz and colleagues suggest that it is this latter phenomenon that may explain the seizures and neurological atrophy seen canonically in MoCD patients [6].

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Patients diagnosed with MoCD are classified into three groups based on which of the three primary steps of Moco biosynthesis is disrupted: Type A – GTP conversion to cPMP, Type B – MPT formation, Type 3 – molybdate insertion to form Moco. Greater than 60% of the identified mutations found in MoCD patients occur in the first step (Type A) of the biosynthetic pathway [105]. Recently, a human patient suffering from Type A MoCD was recently partially cured with treatments of E. coli purified cPMP [109], a therapy that was first pioneered in Moco-deficient mice [110]. Nevertheless, the treatment requires daily injections of the relatively unstable and oxygen-sensitive cPMP, and while no longer fatal, the disease still manifests with significant neurological symptoms. As a result, there is still a pressing need for a greater understanding of MoCD and more effective treatments.
In catabolism of methionine and cysteine, pyruvate and sulfite are generated from the breakdown of the catabolic intermediate, \( \beta \)-sulfinyl pyruvate. In healthy individuals, accumulated sulfite is rapidly oxidized in the mitochondria to sulfate by sulfite oxidase. The loss of activity of the Moco-dependent sulfite oxidase is the primary cause the fatal symptoms associated with Moco deficiency (MoCD). In patients with MoCD, sulfite levels accumulate and combine with cystine to form S-sulfocysteine, a glutamate analog that impairs the healthy activity of the GABA (glutamate) receptor. Additionally, accumulated sulfite levels are though to disrupt ATP synthesis. Catabolic intermediates are indicated in black, and the metabolites and effects of MoCD are highlighted in red. Glutamate is included in the insert for structural comparison to S-sulfocysteine.

Figure 9: Sulfite Oxidation and Molybdenum Cofactor Deficiency
1.4.2.2. Role of the Molybdenum Cofactor in Infectious Disease

Chronic bacterial infections are a significant cause of morbidity and mortality for patients with infectious diseases such as *Mycobacterium tuberculosis* [111, 112] or *Pseudomonas pneumonia* [113]. Frequently, these chronic infections are tolerant to multiple antibiotics, and therefore, require prolonged treatment with multidrug therapies. In the case of chronic and pulmonary tuberculosis, at least 6 months of continuous administration of four different first line drugs is required. In severe cases of *Pseudomonas* infections found in cystic fibrosis patients, surgical removal of the affected area is required. Failure to eradicate these types of chronic infections causes frequent recurrence of symptoms, while the prolonged antibiotic treatment increases the risk of developing multi-drug resistant bacteria [111]. Therefore, chronic bacterial infections are a significant threat to public health.

During chronic infections, bacteria survive under hypoxic and nutrient limiting conditions, where molybdenum cofactor (Moco) biosynthesis is essential. Found in about three quarters of all bacteria, Moco is once again critical for pathogens to maintain their redox function in anaerobic respiration [114]. Without Moco, bacteria cannot sustain sufficient membrane potential under oxygen and nutrient depleted conditions, which eventually leads to cell death. Energy metabolism is a promising target for antibiotics against chronic infections, as the dormant cells require lower but still adequate amounts of ATP to maintain their viability [115].
The requirement for Moco and Moco-dependent enzymes for \textit{in vivo} survival of several important pathogens has been demonstrated in several model systems. In one example, using mouse models for tuberculosis, \textit{Mycobacterium bovis} lacking a Moco-dependent nitrate reductase, an enzyme essential for anaerobic nitrate respiration, was shown to be avirulent and not cause any acute tuberculosis-associated symptoms [116, 117]. Furthermore, the bacteria were cleared from lungs, kidneys, and liver without any additional treatment. Recently, the inhibition of Moco biosynthesis in \textit{M. tuberculosis} by an organic small molecule, TCA1, was shown effective for eradication of latent tuberculosis in mouse models [103]. TCA1 targets two pathways, cell wall and Moco biosynthesis, with the latter shown to be primarily responsible for the drug’s activity against latent tuberculosis. The critical role of Moco has also been demonstrated for the \textit{Pseudomonas} infection. Using a rat lung infection model, a \textit{Pseudomonas aeruginosa} mutant strain with a significant reduction in Moco biosynthesis (5-10\% of wt) was shown to be incapable of replicating in the lung and exhibited significantly reduced virulence [118, 119]. The observed phenotype was rationalized again by reduced activity of nitrate reductase.

All of this evidence strongly suggests the importance of Moco biosynthesis in chronic bacterial infections. Despite this significance, rational development of Moco biosynthesis inhibitors is currently difficult due to the limited understanding in the structures and mechanisms of the biosynthetic enzymes. The only inhibitor available,
TCA1, was identified by a whole-cell high-throughput screening with no bias in Moco synthesis inhibition [103]. Therefore, further understanding of the mechanisms of Moco biosynthetic enzymes in bacteria is essential for future development of Moco biosynthesis inhibitors and the subsequent development of novel therapies that will improve the current treatments against chronic infections.
2. Identification of a Cryptic Nucleotide as a Cryptic Intermediate in Molybdenum Cofactor Biosynthesis

2.1. Introduction

As aforementioned, the molybdenum cofactor (Moco, Figure 10a, 4) is a pterin-based redox cofactor found in almost all organisms [1, 2]. Moco biosynthesis is initiated by the complex transformation of guanosine triphosphate (GTP, Figure 10a, 1), into cyclic pyranopterin monophosphate (cPMP, Figure 10a, 2) [10-12]. cPMP is then converted to Moco by the introduction of two sulfur atoms [13-15] and a molybdate [16-19]. Previous isotope tracer experiments indicated that the conversion of GTP to cPMP proceeds through the insertion of C-8 of guanine between C-2’ and C-3’ of ribose (Figure 10a) [10, 20]. This contrasts with the biosynthesis of pterin rings in other GTP derived cofactors such as folate and flavins, in which C-8 of GTP is released as a formate during their biosynthesis (Figure 11a) [120, 121], as such suggests that Moco biosynthesis utilizes a novel mechanism of pterin ring formation.
Figure 10: Proposed Intermediates for cPMP Formation in Moco Biosynthesis

(a) Overview of Moco biosynthetic pathway in bacteria and humans. Molybdenum cofactor (Moco) biosynthesis is initiated by the formation of cyclic pyranopterin monophosphate (cPMP) from the nucleotide, guanosine triphosphate (GTP), using MoaA and MoaC in bacteria. Subsequently cPMP into Moco through a sulfur insertion and ligation of molybdate. The symbols on GTP and cPMP indicate the source of the carbon and nitrogen atoms in cPMP as determined by isotope labeling studies[10, 20]. P designates a phosphate group. (b) My proposal for cPMP formation occurs through the MoaA product, 3’-8-cH2GTP, which has been identified through the work outlined in this chapter. (c) cPMP formation was previously thought to proceed through an intermediate analogous to other flavin and pterin biosynthetic pathways utilizing GTP Cyclohydrolase. Illustrated here is 2-amino-5-formylamino-6-ribofuranosylamino-5-pyrimidinone triphosphate as an intermediate [4, 77]. (d) In a recent proposal, MoaA is assumed to form the pterin moiety for cPMP, generating a pyranopterin triphosphate product, which is then utilized by MoaC to form cPMP [75, 122].
From the early characterization of the Moco biosynthetic pathway, two enzymes, MoaA and MoaC in bacteria, have been known to be responsible for the conversion of GTP to cPMP [10, 12, 80, 123]. MoaA, as radical SAM (S-adenosyl-L-methionine) enzyme, is theorized to reductively cleave SAM using an oxygen-labile [4Fe-4S] cluster to transiently generate a 5′-deoxyadenosyl radical (5′-dA•), which is directly involved in the reaction chemistry [66]. This was supported experimental by an in vitro activity assay of MoaA in the presence of MoaC, in which the conversion of GTP into cPMP required the presence of SAM [12]. This observation and the aforementioned structural [12] and EPR [72] studies strongly suggest that GTP may be the natural substrate of MoaA.

As many radical SAM enzymes are known to catalyze complex rearrangement reactions [67, 73], MoaA has previously been considered to catalyze the majority, if not all, of the complex rearrangement of GTP to form the pyranopterin ring of cPMP [5, 9, 74, 75]. Schindelin and Hänzelmann first proposed that, in the absence of MoaC, purified MoaA catalyzes a conversion of GTP to a molecule with a 6-hydroxy-2,4,5-triaminopyrimidine partial structure based on the chemical derivatization to dimethylpterin (DMPT, Figure 11b, 20) [71]. Unfortunately, no data were presented. This observation was analogous to those for folate biosynthesis, where the imidazole moiety of guanine base in GTP is first hydrolyzed to an 2-amino-5-formylamino-6-ribofuranosylamino-4-pyrimidinone triphosphate (Figure 11a) [124], which may be
chemically derivatized to DMPT (Figure 11b, 20). This analogy has prompted speculations about the reaction catalyzed by MoaA [74, 76], which consider 2-amino-5-formylamino-6-ribofuranosylamino-4-pyrimidinone triphosphate (Figure 10c, 18) as an intermediate or a product of the MoaA catalyzed reaction (Figure 10c).

Recently, a more definitive proposal for the product of MoaA was made by Begley et al. [75, 122]. In this report, the authors performed LCMS analysis of small molecules produced after incubation of MoaA with GTP, SAM and dithionite in the absence of MoaC, and observed a molecule with a light absorption at 320 nm with a mass signal at $m/z = 524$ [M+H]$^+$ in the presence of GTP, SAM and sodium dithionite. Reactions using deuterated GTP suggested that a deuterium at the 3´ position of GTP is transferred to 5´-deoxyadenosine (5´-dA) produced in this assay. However, the observed putative MoaA product was not isolated for further structural characterization, and the relation of this observation to the earlier one by Schindelin and Hänzelmann [71] was not discussed. It is currently unknown whether the putative MoaA product could serve as a substrate of MoaC, and be converted to cPMP. Nevertheless, based on these observations, the authors proposed that MoaA catalyzes a conversion of GTP into pyranopterin triphosphate (Figure 10d, 19) [75, 122].

The role of MoaC, on the other hand, has been significantly underappreciated. Once again, MoaC does not show significant amino acid sequence similarities to functionally characterized proteins, and while several apo-structures of MoaC have been
solved from four different organisms [76-79], only a putative ligand-binding site has ever been proposed. In the recent publication by Begley et al. [75], MoaC was suggested to be responsible only for the formation of the cyclic phosphate (Figure 10d), based on their premise that the MoaA product is pyranopterin triphosphate. However, uncertainty remains about the relevance of this proposal due to the limited characterization of the MoaA reaction product.

Figure 11: 6-hydroxy-2,4,5-triaminopyrimidine Nucleotides in Pterin and Flavin Biosynthesis

(a) The reactions catalyzed by GTP cyclohydrolases (GTPCH) in the biosynthesis of pterins and flavins. (b) Derivatization of 6-hydroxy-2,4,5-triaminopyrimidine nucleotides to DMPT.
In this current chapter, I report the isolation and detailed characterization of the MoaA reaction product, which unambiguously delineated the individual reactions catalyzed by MoaA and MoaC. The isolation of the MoaA reaction product was achieved under anaerobic conditions with careful control of pH. Structural characterization by chemical derivatization, MS, and NMR spectroscopy established the structure of this molecule as (8S)-3’,8-cyclo-7,8-dihydroguanosine 5’-triphosphate (3’,8-cH2GTP, Figure 10b, 17). The relevance of the isolated 3’,8-cH2GTP as a physiological Moco biosynthetic intermediate was demonstrated by steady state kinetic analysis of MoaC and its human homolog, MOCS1B. MoaC and MOCS1B converted 3’,8-cH2GTP to cPMP with \( K_m \) values of < 0.06 \( \mu \)M and 0.79 \( \mu \)M, respectively. Additional studies on the stoichiometry of the MoaA reaction in combination with an isotope tracer experiment suggest that the conversion of GTP to 3’,8-cH2GTP proceeds through H-abstraction from the 3’ position by consuming a stoichiometric amount of SAM. The observations presented here in sum unequivocally delineate the individual functions of MoaA and MoaC, and provide insights into the biosynthesis of cPMP. The current identification of 3’,8-cH2GTP as a substrate of MoaC is a sharp contrast to previous proposals [74, 75], in which MoaC was thought to have minimal or no function in the formation of the pyranopterin structure. These results provided the basis for future mechanistic studies of reactions catalyzed by these two enzymes.
2.2 Results

2.2.1. Investigation for an Intermediate Between GTP and cPMP

While small molecules produced by MoaA were previously proposed to serve as a substrate of MoaC [75], the conversion of this molecule to cPMP by MoaC in the absence of MoaA has never been demonstrated. Considering the complexity of the conversion of GTP into cPMP, and enzyme-catalyzed radical reactions in general [73, 125], the absence of such demonstration leaves significant ambiguity about the relevancy of their observations to Moco biosynthesis. Thus, I carried out stepwise activity assays using *Staphylococcus aureus* MoaA and MoaC. In these assays, GTP (1 mM) was first incubated with MoaA (5 µM), SAM (1 mM) and sodium dithionite (1 mM) in the absence of MoaC. The resulting small molecules were separated from MoaA by ultrafiltration, and subsequently incubated with MoaC (5.6 µM). Any cPMP present in the resulting reaction solution was quantified by HPLC analysis after a conversion to its fluorescent derivative, compound Z (CmdZ, Figure 13a; 6), following the previously established protocol [11]. Figure 13c shows the results of HPLC analysis that shows a peak co-migrating with compound Z. Quantitation of the observed HPLC peak using an authentic standard suggested formation of 3.6 ± 0.3 µM compound Z, substoichiometric to MoaA. Control reactions lacking GTP or SAM did not yield compound Z. These observations are consistent with the presence of a small molecule intermediate that is produced by MoaA, and converted to cPMP by MoaC.
2.2.2. Chemical Derivatization of the MoaA Reaction Product

For the biosynthesis of other pterins and flavins, nucleotide biosynthetic intermediates with a 6-hydroxy-2,4,5-triaminopyrimidine partial structure have been reported [124, 126]. These nucleotides have been characterized by derivatization of a hydrolytically released 6-hydroxy-2,4,5-triaminopyrimidine to the highly fluorescent DMPT. Schindelin et al. briefly mentioned in their publication [71] that the MoaA reaction product can be converted to DMPT, although no data were presented. I thus attempted a similar derivatization to investigate whether the MoaA product contains an acid-labile 6-hydroxy-2,4,5-triaminopyrimidine partial structure. For this purpose, enzyme activity assays were performed with MoaA (5 µM), GTP (1 mM), SAM (1 mM) and sodium dithionite (1 mM) under anaerobic conditions in the absence of MoaC. The
reaction was stopped by the addition of hydrochloric acid, and the resulting solution was incubated at 95 °C to facilitate hydrolysis, followed by incubation with 2,3-butanedione at pH 8. Figure 13c shows the HPLC analysis of this reaction, which reveals the formation of DMPT. Omission of any of the reaction components resulted in significant decrease of DMPT. The time course assay revealed that increasing amounts of DMPT were formed with prolonged incubation time (Figure 13d). The concentration of DMPT observed in this assay at the 60 min time point (3.8 ± 0.2 μM) was substoichiometric to that of MoaA (5 μM), and within errors to the amount of cPMP observed in the stepwise assay (3.6 ± 0.3 μM, compare filled and open circles at 60 min time point in Figure 13d). Together with the results of the stepwise assays, these preliminary characterizations prompted us to attempt isolation of 3’,8-cH2GTP.
(a) 

GTP (1) → MoaA → Intermediate (3',8-chGTP, 17) → MoaC → cPMP (2) → DMPT (20) → Compound Z (6)

1. pH 2.0, Heat
2. 2,3-butanedione

(b) (c)  

Relative fluorescent intensity

Retention time (min)

(d)  

Compound Z or DMPT (µM)

Time (min)
Figure 13: In vitro Stepwise Activity Assay of MoaA and MoaC

(a) Overview of chemical derivatization of in vitro stepwise activity of MoaA and MoaC 
(b) HPLC analysis of MoaA activity assay (Ex. 365 nm, Em. 445 nm). In the complete condition, MoaA (5 μM), was incubated with GTP (1 mM), SAM (1 mM) and sodium dithionite (1 mM) at 25 °C for 60 min. The reaction product was subjected to acid hydrolysis, followed by incubation with 2,3-butanedione, and analyzed for the fluorescent DMPT. Also shown are the chromatograms for the DMPT standard, and for control reactions lacking GTP, SAM, dithionite, or MoaA. A small amount of DMPT (~10% of the complete condition) was formed in the GTP negative control due to the co-purification of GTP with MoaA. 
(c) HPLC analysis of stepwise MoaA/MoaC activity assay (Ex. 367 nm, Em. 450 nm). In the complete reaction, GTP (1 mM), SAM (1 mM) and sodium dithionite (1 mM) were incubated with MoaA (5 μM) for 60 min at 25 °C, followed by removal of MoaA by ultrafiltration and incubation with MoaC (15 μM) for 60 min at 25 °C. cPMP was converted to compound Z (6), and detected by HPLC. Also shown are the chromatograms for the compound Z standard, and control reactions without GTP or SAM. 
(d) Time-course of 3’,8-ch2GTP formation determined after conversion to DMPT (filled circles) or compound Z (open circle). The conditions for the MoaA reaction and the derivatization of the product to DMPT are identical to (b). The amount of compound Z at 60 min is based on the quantitation of the HPLC peak in (a). Each point is an average of three replicates, and the error bars are calculated based on the standard deviation.

2.2.3. Isolation of 3’,8-ch2GTP

The isolation of 3’,8-ch2GTP was a significant challenge primarily due to its limited stability. As shown in Figure 3a, decomposition of 3’,8-ch2GTP was observed under aerobic conditions or under acidic pH. This limited stability required all the purifications to be carried out in an anaerobic glove box (O2 concentration < 0.1 ppm) maintained at 10 °C using ammonium bicarbonate buffer at pH 9.1. Under these conditions, no decomposition was observed at least for 3 h (Figure 14a).

MoaA reaction conditions were chosen to maximize the production of 3’,8-ch2GTP, as well as the percentage conversion of GTP to 3’,8-ch2GTP. It was important
to consume as much GTP as possible in the MoaA reaction because of the close separation between GTP and 3',8-cH$_2$GTP during purification with anion exchange resin. The reaction is limited by the substoichiometric turnover of MoaA. The maximum turnover observed after 60 min of incubation at 25 °C was 0.5 ± 0.2 when stoichiometric or excess amount of GTP relative to MoaA was used (Figure 13d). The use of substoichiometric amount of GTP to MoaA significantly lowered the yield. Thus, for the isolation of 3',8-cH$_2$GTP, GTP concentration was kept stoichiometric relative to MoaA.

**Figure 14: Stability of MoaA product, 3',8-cH$_2$GTP**

(a) Stability of 3',8-cH$_2$GTP under different conditions. Solution containing 3',8-cH$_2$GTP was incubated for 10-180 min under specified conditions. At each time point, an aliquot was removed and 3',8-cH$_2$GTP was quantified by HPLC after its conversion to compound Z. Each point is an average of three replicates, and the error bars are calculated based on the standard deviation. (b) UV-vis absorption spectrum of 3',8-cH$_2$GTP (bold trace) (60 µM). The thin traces are spectra after exposure to air at 22 °C for the specified time.

Purification conditions also required careful optimization to avoid decomposition. Successful purification was achieved by the use of two anion exchange columns (Figure 15a). A strong anion exchange (QAE sepharose) resin was used as the
first step of purification to process a large volume of sample and to separate 3’,8-ch2GTP and GTP from the other components in the MoaA assay mixture. A weak anion exchange (DEAE sepharose) resin was used to separate GTP from 3’,8-ch2GTP (Figure 15b). Typically, 10 µmol of 3’,8-ch2GTP was isolated from 200 mL of enzyme reaction mixture containing 0.2 µM MoaA, 0.2 mM GTP, 1.0 mM SAM and 1.0 mM sodium dithionite. The isolated 3’,8-ch2GTP had a unique absorption feature at 322 nm (Figure 14b, bold trace). This absorption feature also exhibited oxygen sensitivity and diminished upon exposure to air (Figure 14b, thin traces), consistent with the oxygen sensitivity of 3’,8-ch2GTP observed by the biochemical characterization (Figure 14a).

The isolated 3’,8-ch2GTP was also analyzed for the number of phosphates in the molecule, as its close migration with GTP on the anion exchange resin suggested the presence of a triphosphate group. The use of a colorimetric phosphate assay [127] after a phosphatase treatment revealed 2.93 ± 0.03 phosphate groups per molecule. This result is consistent with 3’,8-ch2GTP being a triphosphate compound.
Figure 15: *In vitro* Purification of MoaA Product, 3’,8-cH₂GTP

(a) Overview of *in vitro* purification scheme for 3’,8-cH₂GTP using anaerobic anion exchange chromatography. Chromatograms of 3’,8-cH₂GTP purification on QAE sepharose (b), and DEAE sepharose (c) column chromatography. Elution from each column chromatography was performed by a linear gradient increase of the ammonium bicarbonate (pH 9.0) concentration indicated by the dotted lines. Each fraction was assayed for GTP by UV absorbance at 256 nm (black dashed trace), or for 3’,8-cH₂GTP by UV absorbance at 322 nm (grey dashed trace) or by conversion to compound Z (solid black trace).

2.2.4. Molecular Weight Determination of 3’,8-cH₂GTP

An ESI-TOF-MS analysis of isolated 3’,8-cH₂GTP revealed \( m/z = 521.984 \pm 0.001 \) [M-H] (Figure 16a), consistent with the molecular formula of C₁₀H₁₅N₅O₁₄P₃ (calcd. \( m/z = 521.983 \) [M-H]). The observed mass was also consistent with GTP, which migrates close to 3’,8-cH₂GTP during the anion exchange chromatographies (see Figure S3), and occasionally contaminated 3’,8-cH₂GTP. Thus, it was important to verify that the observed signal was indeed associated with 3’,8-cH₂GTP rather than contaminating
GTP. To distinguish 3′,8-cH₂GTP from GTP, purified 3′,8-cH₂GTP was incubated with MoaC and analyzed by MS, which exhibited no MS signal at \( m/z = 521.984 \), and instead exhibited a signal at \( m/z = 362.052 \pm 0.001 \) [M-H]⁻ (Figure 16b) corresponding to a hydrate form of cPMP (\( m/z \) [M-H]⁻ calcd. for \( \text{C}_{10}\text{H}_{13}\text{N}_{5}\text{O}_{8}\text{P} \), 362.051). These findings suggest that the observed MS signal was indeed associated with the intact 3′,8-cH₂GTP, but not contaminating GTP.

(a) ESI-TOF-MS analysis of 3′,8-cH₂GTP. The times for the pre-incubation of 3′,8-cH₂GTP in air are indicated. The signal at \( m/z = 442.109 \) corresponds to the 3′,8-cyclo-7,8-dihydroguanosine 5′-diphosphate (3′,8-cH₂GDP, \( m/z \) [M-H]⁻ calcd. for \( \text{C}_{10}\text{H}_{14}\text{N}_{5}\text{O}_{11}\text{P} \), 442.017). (b) ESI-TOF-MS analysis of 3′,8-cH₂GTP pre-incubated with MoaC. The sample was prepared by anaerobic incubation of 3′,8-cH₂GTP (100 µM) with MoaC (50 µM) in 300 mM ammonium bicarbonate pH 9.1 (400 µL) for 20 min at 25 °C. After removal of MoaC by ultrafiltration (Amicon Ultra 10 K, Millipore) in the glovebox, the small molecule fraction was lyophilized and redissolved in anaerobic water (400 µL). The concentration of cPMP in this sample was 100 µM judged by the chemical conversion to compound Z and quantitation by HPLC as described for the 3′,8-cH₂GTP Stability Test.
2.2.5. Structural Characterization of 3´,8-cH₂GTP by NMR Spectroscopy

Further structural characterization of 3´,8-cH₂GTP was carried out using NMR spectroscopy. Figure 17a shows the ¹H NMR spectrum of purified 3´,8-cH₂GTP with six signals between 4.1 – 5.3 ppm. To confirm that these signals are associated with 3´,8-cH₂GTP, the observed ¹H NMR signals were quantified by comparing the integrals of the observed signals with that of a known concentration of maleic acid as an internal standard. The concentration of 0.59 ± 0.14 mM was determined by this analysis, which agreed well with the concentration (0.67 ± 0.03 mM) biochemically determined after its conversion to compound Z.

The ¹³C NMR spectrum (Figure 17b) was recorded on 3´,8-cH₂GTP uniformly labeled with ¹³C and ¹⁵N, prepared from [U-¹³C₁₀,¹⁵N₅]GTP. Four carbon signals were found at 111, 154, 156, and 160 ppm, suggesting the presence of four sp² carbons. The major multiplicities of the ¹³C NMR signals (Figure 17b) are derived from ¹J₁³C-¹³C coupling. The analysis of the multiplicities (Figure 17b and Table 1) in combination with the 2D NMR spectral correlations (Figures 19-24) established the connectivities among the signals at 111, 156, and 160 ppm. Together with the comparison with the chemical shifts reported for various purines and pyrimidines (Table 2) [128, 129], I assigned the four sp² carbon signals to the carbons in the 6-hydroxy-2,4,5-triaminopyrimidine moiety of 3´,8-cH₂GTP.
Figure 17: 1D NMR Structural Characterization of 3’,8-cH₂GTP

(a) ¹H NMR (600 MHz, D₂O) and (b) ¹H-decoupled ¹³C NMR (600 MHz, D₂O) spectra of 3’,8-cH₂GTP and [U-¹³C₁₀,¹⁵N₅]3’,8-cH₂GTP, respectively. No signal associated with 3’,8-cH₂GTP was observed outside the shown chemical shift range. The intensity of the H-1’ signal in (a) was decreased due to the water suppression at 4.95 ppm. The insets in (b) shows magnified view of the signals for C-3’, C-4’ and C-8. (c) Direct ¹³C NMR characterization of the MoaA reaction using [U-¹³C₁₀,¹⁵N₅]GTP as the substrate. Shown are ¹³C NMR spectra of [U-¹³C₁₀,¹⁵N₅]GTP, isolated [U-¹³C₁₀,¹⁵N₅]3’,8-cH₂GTP, and the MoaA assay solution. The MoaA assay was performed in an anaerobically sealed NMR tube using MoaA, SAM, [U-¹³C₁₀,¹⁵N₅]GTP, sodium dithionite in Tris-HCl pH 7.6 at 25 °C.
for 1 hr. The resulting sample was subjected to $^{13}$C NMR analysis without any work-up. Signals highlighted by * and # are derived from toluenesulfonate and glycerol, respectively.

The remaining $^{13}$C NMR signals were found between 63.7 and 90.8 ppm with observed $^{13}$C-$^{13}$C coupling constants of ~40 Hz, suggesting that they are all sp$^3$ carbons [130]. The analysis of the multiplicities in combination with the $^{13}$C-$^{13}$C COSY correlations (Figure 20) unambiguously determined the C-C connectivities as shown in Figure 18a. The most notable is the presence of C-3’ as a tertiary carbon that appeared as a quartet signal in the $^{13}$C NMR spectrum (see the inset of Figure 17b). The connectivity of C-3’ to C-8 in addition to C-2’and C-4’ was established by the $^{13}$C-$^{13}$C COSY correlations. This feature distinguishes this molecule from GTP, cPMP, or molecules that have been previously proposed as MoaA products; 2-amino-5-formylamino-6-ribofuranosylamino-4-pyrimidinone triphosphate (18) and pyranopterin triphosphate (19) [74, 75], because none of these molecules has a tertiary carbon. This analysis suggested that during the MoaA reaction, the C3’-C8 bond is formed, but the pyranopterin structure is not yet established.

Further NMR analysis provided basis for the complex multi-ring structure around C-8. $^1$H-$^{13}$C HMQC (Figure 21), multiplicity of the $^{13}$C NMR signal (Figure 17b) and the $^{13}$C-$^{13}$C COSY correlation (Figure 20) suggested that C-8 is a methine primary carbon. The $^1$H-$^{13}$C HMBC correlations from H-8 to C-1’, and from H-1’ to C-8 (Figure 18a) suggested a connection between C-8 and C-1’ through a heteroatom. The $^1$H-$^{15}$N
HMBC correlations to the same N from H-1´ and from H-8 (Figure 18a) suggested that the bridging heteroatom is N. The second heteroatom on C-8 would be either N or O. The reported chemical shift values for methine primary carbons covalently bound to an N and an O are 84-97 ppm [124, 131-133], whereas those with two Ns are 71-86 ppm [132, 134-137]. Thus, the chemical shift observed for C-8 (75.6 ppm) is most consistent with the presence of the second N on C-8.

Table 1: Summary of NMR data for 3´,8-CH2GTP

<table>
<thead>
<tr>
<th>No.</th>
<th>C δ(ppm) (m, $J_{CC}$(Hz))</th>
<th>H δ(ppm) (m, $J_{HH}$(Hz))</th>
<th>1H-1H COSY</th>
<th>13C-13C COSY</th>
<th>1H-13C HMBC</th>
<th>1H-15N HMBC</th>
<th>NOESY</th>
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<td>2</td>
<td>153.6, (s)</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
<td>156.1, (d, 83.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C-5</td>
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<tr>
<td>5</td>
<td>110.8 (t, 76.0)</td>
<td>C-4, C-6</td>
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<tr>
<td>6</td>
<td>160.0 (d, 68.0)</td>
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<tr>
<td>8</td>
<td>75.6 (d, 42.5)</td>
<td>5.20 (s)</td>
<td>C-3´</td>
<td>H-1´, H-4´</td>
<td>N-9</td>
<td>H-5a</td>
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<td>1´</td>
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<td>5.02 (s)</td>
<td>C-2´</td>
<td>H-2´, H-8</td>
<td>N-9</td>
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<tr>
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<td>4.14 (s)</td>
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<td>4.48 (dd, 3.9, 7.3)</td>
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<tr>
<td>5´a</td>
<td>63.7 (d, 44.9)</td>
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<td>5´b</td>
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*The doublet feature is derived from a $J_{1H-1H}$ to the geminal $1H$, and the triplet feature is from a $J_{1H-1H}$ to H-4´ and a $J_{31P-1H}$ to $31P$ of the phosphate group.
Figure 18: Summary of Structural Characterization of 3',8-cH₂GTP

(a) $^{13}$C-$^{13}$C COSY (bold), and selected $^1$H-$^{13}$C, and $^1$H-$^{15}$N HMBC (single-headed arrows) correlations, and NOE (a double-headed arrow). A complete list of the observed correlations is available in Table 1. P designates a phosphate group. (b) Energy minimized conformation of the nucleoside moiety of 3',8-cH₂GTP. MM2 calculation was performed on ChemDraw 3D (Cambridgesoft). Carbon (gray balls), nitrogen (blue balls), oxygen (red balls), and hydrogen (white balls) atoms and lone pairs (pink balls) are shown. The dihedral angle for H1'-C1'-C2'-H2' is also indicated. (c) Possible orientations of 6-hydroxy-2,4,5-triaminopyrimidine. The structure I propose for 3',8-cH₂GTP is highlighted in blue.

The connections of C-1' and C-8 to nitrogen atoms suggested the connections to 6-hydroxy-2,4,5-triaminopyrimidine because all nitrogen atoms are associated with the 6-hydroxy-2,4,5-triaminopyrimidine based on the molecular formula determined by MS.
The connection to 6-hydroxy-2,4,5-triaminopyrimidine is supported by the observation of a weak $^1$H-$^{13}$C HMBC correlation from H-8 to C-6 (Figures 18a, and 22). While several different orientations for 6-hydroxy-2,4,5-triaminopyrimidine are consistent with the NMR data (Figure 18c), the nitrogen bridging C-8 and C-1´ is likely N-9, considering that the previous isotope tracer experiments indicated that the N9-C1´ bond is maintained during the transformation of GTP into cPMP [10, 20]. The second N on C-8 was assigned as N-7, considering that C8-N7 bond is present in GTP, and there is no evidence suggesting that the other Ns are involved in the transformation of GTP to cPMP. Based on these analyses, I propose the orientation of the 6-hydroxy-2,4,5-triaminopyrimidine connection as shown in Figure 18a.

The structures of the remaining part of the molecule were based on the following analysis. The furanose ring was based on the $^1$H-$^{13}$C HMBC correlations from H-1´ to C-4´ (Figure 18a). The position of the triphosphate group was based on the $^3$J$_{HP}$ = 5 - 6 Hz observed for the $^1$H NMR signals for H-5´a and H-5´b (Figure 18a, and Table 1), which are comparable to those reported for various nucleotides [138, 139]. The hydroxyl groups at the 2´ and 3´ positions were based on the NMR chemical shifts observed for H-2´, C-2´, and C-3´. The stereochemistry at C-1´, C-2´, C-3´, and C-4´ is based on GTP and cPMP [87]. The absence of an apparent J coupling between H-1´ and H-2´ ($^3$J$_{H1'H2'}$ < 4 Hz) is consistent, based on the Karplus relations [140], with the calculated dihedral angle of 59 ° between H1´-C1´-C2´-H2´ (see Figure 18b for the energy minimized conformation of
3’,8-cH₂GTP). The stereochemistry at C-8 is based on NOE observed between H-8 and H-5’a (Figure 18a; a double headed arrow).

Based on all the analysis described above, I propose the structure of the isolated molecule as (8S)-3’,8-cyclo-7,8-dihydroguanosine 5’-triphosphate (3’,8-cH₂GTP, Figure 18a). The proposed structure is consistent with all the NMR data (Table 1), as well as MS, phosphate assay and chemical derivatization to DMPT described above.

In order to address the continuing argument about the product of MoaA, I employed in situ observation of the MoaA reaction using ¹³C-labeled GTP as a substrate in combination with ¹³C NMR as the method of detection. This method allows direct monitoring of the MoaA reaction without any treatment that could potentially alter the chemical structure of the unstable MoaA product. In this experiment, MoaA assay was performed in an anaerobically sealed NMR tube, which was directly subjected to ¹³C NMR analysis. Figure 17c shows the comparison of the ¹³C NMR spectra of MoaA assay solution, [U⁻¹³C]GTP and purified [U⁻¹³C]3’,8-cH₂GTP. The spectra of the MoaA assay solution can be simulated using the spectra of [U⁻¹³C]GTP and [U⁻¹³C]3’,8-cH₂GTP in a x : y ratio. This ratio is consistent with the amount of GTP and 3’,8-cH₂GTP determined based on the chemical derivatization method. No signal that could be interpreted as pyranopterin structure is observed [141]. Therefore, these observations strongly support that 3’,8-cH₂GTP is the only detectable product of MoaA. Previous observation of
pyranopterin-like molecule is likely an artifact of complex chemoenzymatic derivatization [75, 122, 142].

Figure 19: $^1$H-$^1$H COSY spectrum of 3',8-CH₂GTP
Figure 20: $^{13}$C-$^{13}$C COSY spectrum of [U-$^{13}$C$_{10}$, $^{15}$N$_{5}$]3’-$\delta$-CH$_{2}$GTP
Figure 21: $^1\text{H}-^{13}\text{C}$ HMQC spectrum of $3',8$-cH$_2$GTP
Figure 22: $^1$H-$^{13}$C HMBC spectrum of 3',8-CH$_2$GTP
Figure 23: $^1$H-$^{15}$N HMBC spectrum of [U-$^{13}$C$_{10}$,$^{15}$N$_5$]3',8-$\text{cH}_2$GTP
Figure 24: NOESY spectrum of 3',8-cH$_2$GTP
2.2.6 The Relevance of 3´,8-cH₂GTP to Moco Biosynthesis

The physiological relevance of 3´,8-cH₂GTP as an intermediate of Moco biosynthesis was further investigated by steady state kinetics of the MoaA and MoaC catalyzed reactions. The kinetic parameters for the MoaC catalyzed conversion of 3´,8-cH₂GTP to cPMP were determined using purified 3´,8-cH₂GTP as the substrate. The kinetic parameters for the MoaA catalyzed reaction were determined by a coupled assay in the presence of a 10-fold excess of MoaC to ensure efficient conversion of 3´,8-cH₂GTP to cPMP. In both assays, cPMP was converted to compound Z and quantified by HPLC.

Figure 25: ¹³C NMR Chemical Shifts for Purine and Pyrimidine Bases

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<th>Position</th>
<th>3´,8-cH₂GTP</th>
<th>G⁶</th>
<th>8-oxo-G⁷</th>
<th>8-OMe-G⁷</th>
<th>8-Br-G⁷</th>
<th>8-Me-G⁷</th>
<th>A⁸</th>
<th>C⁸</th>
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<td>156.5</td>
<td>156.4</td>
<td>165.5</td>
<td>163.6</td>
<td>163.0</td>
<td>163.9</td>
</tr>
<tr>
<td>C8</td>
<td>75.6</td>
<td>136.0</td>
<td>151.8</td>
<td>151.6</td>
<td>121.3</td>
<td>145.1</td>
<td>140.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Solvent: D₂O, DMSO

*Current study.

Chemical shifts are shown in ppm for G⁶, 8-oxo-G⁷, 8-OMe-G⁷, 8-Br-G⁷, 8-Me-G⁷, A⁸, C⁸, T⁸, U⁸, and IsoC⁸.
The results are summarized in Table 2. The $k_{\text{cat}}$ of MoaC was ~4-fold greater than that of MoaA determined by this method, and the $K_m$ of MoaC for 3′,8-cH$_2$GTP was < 0.06 $\mu$M, the lower limit of detection. $K_m$ of MoaA for GTP was found to be 3.1 ± 0.7 $\mu$M, which may be compared to the previously reported binding constant of 0.29 ± 0.11 $\mu$M determined by an equilibrium dialysis [71]. These observations support the conclusion that 3′,8-cH$_2$GTP is likely the physiological substrate of MoaC, and the biosynthetic intermediate between GTP and cPMP during Moco biosynthesis.

**Table 2: Steady state kinetic parameters for MoaA, MoaC, and MOCS1B**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ ($\mu$M)</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$k_{\text{cat}}/K_m$ ($\mu$M$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MoaA</td>
<td>GTP</td>
<td>3.1 ± 0.67</td>
<td>0.042 ± 0.005</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>SAM</td>
<td>5.1 ± 1.4</td>
<td>0.045 ± 0.007</td>
<td>0.0088</td>
</tr>
<tr>
<td>MoaC</td>
<td>3′,8-cH$_2$GTP</td>
<td>&lt;0.060</td>
<td>0.394 ± 0.005</td>
<td>&gt;6.56</td>
</tr>
<tr>
<td>MOCS1B</td>
<td>3′,8-cH$_2$GTP</td>
<td>0.79 ± 0.18</td>
<td>0.092 ± 0.007</td>
<td>0.011</td>
</tr>
</tbody>
</table>

The relevance of 3′,8-cH$_2$GTP as an intermediate in human Moco biosynthesis was investigated by the characterization of a human homolog of MoaC. Previous gene complementation studies suggested that the human proteins, MOCS1A and MOCS1B, have functions that correspond to those of MoaA and MoaC, respectively [83]. In human cells, MOCS1B is expressed as an N-terminal fusion with a catalytically inactive MOCS1A [83, 123]. Since I have not been successful in obtaining the fusion protein, the stand-alone MOCS1B domain was expressed as an N-terminal His-tag protein, and
purified by Ni-affinity chromatography. The HPLC analysis of the product of the MOCS1B activity assay with 3′,8-cH₂GTP as the substrate revealed that cPMP was formed with the kinetic parameters of $K_m = 0.79 \pm 0.24 \mu M$, and $k_{cat} = 0.092 \pm 0.020 \text{ min}^{-1}$, which are somewhat less efficient than, but comparable to $K_m$ and $k_{cat}$ determined for MoaC (Table 2). While these kinetic parameters could be affected by the observed truncation at the N-terminus and/or the lack of the MOCS1A domain, the results show that 3′,8-cH₂GTP is a substrate for MOCS1B, and thus suggest that 3′,8-cH₂GTP may be a biosynthetic intermediate in human Moco biosynthesis as well.
Figure 26: Steady state kinetic analysis of MoaA, MoaC, and MOCS1B.

(a) The rate of MoaA catalysis at various GTP concentrations. The assay was carried out with MoaA (0.5 µM), MoaC (5 µM), SAM (1 mM), and GTP (0.25, 0.50, 1.0, 2.0, 5.0, 10.0, 50.0, 100, 175, 250 µM). A non-linear fit to the Michaelis-Menten equation with $k_{\text{cat}} = 0.042 \text{ min}^{-1}$, and $K_m = 3.1 \mu M$ is shown. (b) The rate of MoaA catalysis at various SAM concentrations. The assay was carried out with MoaA (1 µM), MoaC (10 µM), GTP (1 mM), and SAM (1, 5, 10, 50, 100, 175 or 250 µM). A non-linear fit to the Michaelis-Menten equation with $k_{\text{cat}} = 0.045 \text{ min}^{-1}$, and $K_m = 5.1 \mu M$ is shown. (c) The rates of MoaC catalysis at various 3’,8-cH₂GTP concentrations. The assay was carried out with MoaC (0.1 µM), and 3’,8-cH₂GTP (0.06, 0.17, 0.57, 1.7, 5.7, or 6.0 µM). A non-linear fit to the Michaelis-Menten equation with $k_{\text{cat}} = 0.17 \text{ min}^{-1}$, and $K_m = 0.01 \mu M$ is shown. (d) The rates of MOCS1B catalysis at various 3’,8-cH₂GTP concentrations. The assay was carried
out with MOCS1B (0.5 µM), and 3´,8-cH₂GTP (0.1, 0.2, 0.35, 0.5, 1.0, 3.0, or 6.7 µM). A non-linear fit to the Michaelis-Menten equation with $k_{\text{cat}} = 0.092 \text{ min}^{-1}$, and $K_m = 0.79 \mu M$ is shown. All data are averages of two or three replicates, and error bars are the standard deviations among the replicates.

2.2.7. Stoichiometry of the MoaA Reaction and the Position of H-atom Abstraction

Since MoaA is a member of the radical SAM superfamily, the conversion of GTP to 3´,8-cH₂GTP is likely initiated by a radical formation at C-3´ by 5´-dA• formed by reductive cleavage of SAM, followed by the attack of C-3´ to C-8. In the reactions catalyzed by radical SAM enzymes, SAM may be consumed as a co-substrate, or regenerated and used as a co-catalyst [67]. Since the conversion of GTP into 3´,8-cH₂GTP could take place either of the two mechanisms, I quantified the amount of 5´-dA and cPMP formed in the MoaA/MoaC coupled assay. A 2-fold excess of MoaC was used relative to MoaA to ensure complete conversion of 3´,8-cH₂GTP to cPMP. HPLC quantitation of 5´-dA suggested that, under this condition, stoichiometric amounts of 5´-dA and 3´,8-cH₂GTP were formed (Figure 27a).

The determination of the stoichiometry of SAM cleavage for radical SAM enzymes has been frequently obscured by abortive production of 5´-dA [143]. While my data are consistent with stoichiometric formation of 5´-dA, it is important to show that the observed 5´-dA is indeed the product of H-atom abstraction from GTP, but not a product of abortive SAM cleavage. Thus, I investigated the transfer of a deuterium atom on GTP into 5´-dA. Based on the structure of 3´,8-cH₂GTP, the reaction most likely proceeds through H-abstraction from the 3´ position. Thus, [3´-²H]GTP was
chemoenzymatically prepared from [3-²H]ribose, and used to investigate a deuterium transfer from [3′-²H]GTP to 5′-dA. When [3′-²H]GTP was used as a substrate, a small but significant kinetic isotope effect of $V_{H}/V_{D} = 1.28 \pm 0.05$ was observed (Figure 27b), which is comparable to a value previously reported for another radical SAM enzyme, BtrN ($V_{H}/V_{D} = 1.3 \pm 0.1$) [144].

To investigate the H-3′ transfer from GTP to 5′-dA, 5′-dA was isolated from a large scale MoaA/MoaC coupled reaction mixture, where [3′-²H]GTP (98 µM) was incubated with MoaA (50 µM), MoaC (100 µM), SAM (1 mM), and sodium dithionite (1 mM) at 25 °C for 60 min. HPLC analysis of this assay solution revealed formation of 48 ± 2 µM of 5′-dA and 47 ± 2 µM of cPMP. 5′-dA was isolated using reverse phase silica-gel column chromatography and HPLC, and characterized by MS (Figure 27c), and ¹H and ²H NMR spectroscopies (Figure 27d). These analyses revealed incorporation of a single deuterium atom in the 5′-methyl group of 5′-dA, consistent with a recent observation by Begley’s group [75]. Under the conditions I investigated, ~30% of 5′-dA was formed with no deuterium incorporation (Figure 27c). I attribute this at least partially to the incomplete labeling of [3′-²H]GTP (94 ± 3% atom ²H), and the presence of non-labeled GTP copurified with MoaA. The amount of co-purified GTP was determined as 16 ± 1% that of MoaA by HPLC after its release from MoaA by acid denaturing. Together with the ~50% conversion of [3′-²H]GTP to cPMP, these observations suggest that majority of the non-labeled 5′-dA formed in this reaction was originated from the non-labeled GTP.
in the sample. Thus, my observation is consistent with a mechanism, in which MoaA catalyzes H-abstraction from the 3’ position of GTP by stoichiometric consumption of SAM.

Figure 27: Mechanistic Studies on the MoaA-catalyzed H-atom Abstraction

(a) Stoichiometry of formation of 5’-dA (open circles) and cPMP (filled circles) in the MoaA/MoaC coupled assay. MoaA (20 µM) was incubated with GTP (1 mM), SAM (1 mM) and sodium dithionite (1 mM) in the presence of MoaC (40 µM) at 25 °C. cPMP
was quantified by HPLC after its conversion to compound Z. Each point is an average of 3-6 replicates, and the error bars are calculated based on the standard deviation. (b) Rate of cPMP formation from GTP or [3'-2H]GTP by MoaA and MoaC. MoaA (0.5 μM) was incubated with GTP or [3'-2H]GTP (0.1 mM) in the presence of sodium dithionite (1 mM), SAM (1 mM) and MoaC (5 μM) at 25 °C. cPMP was quantified by HPLC after its conversion to compound Z. Each point is an average of four replicates, and the error bars are calculated based on the standard deviation. (c) ESI-TOF-MS and (d) 1H NMR (600 MHz in D2O) and 2H NMR (600 MHz in H2O) spectra of 5'-dA isolated from the reaction in the presence of MoaA (50 μM), MoaC (100 μM), SAM (1 mM), [3'-2H]GTP (98 mM, 94 ± 3 % atom 2H) and sodium dithionite (1 mM). The signal indicated with (*) is non-deuterated 5'-dA (m/z [M+H]+ calcd. for C16H31N5O3 252.110; found 252.111) presumably derived from non-labeled GTP (see main text).

2.3. Discussion

This chapter describes the isolation and structural characterization of 3',8-cH₂GTP, a previously unidentified intermediate in Moco biosynthesis. My stepwise assay indicated that there is a small molecule that is produced by MoaA in the presence of GTP, SAM and dithionite, and that may serve as a substrate of MoaC. This compound was isolated from the in vitro MoaA assay mixture using two sequential anion exchange column chromatographies under strict anaerobic conditions and structurally characterized by chemical derivatization, MS, and NMR spectroscopy. The physiological relevance of the isolated 3',8-cH₂GTP was investigated by the steady state kinetic analysis of MoaC or its human homolog, MOCS1B. In these analyses, both enzymes catalyze the conversion of 3',8-cH₂GTP to cPMP with high specificities (K_m values of < 0.060 and 0.79 μM, respectively). Although the observed turnover rate for MoaA (kcat = 0.053 min⁻¹), and MoaC (kcat = 0.17 min⁻¹) were slow, they are typical for enzymes in cofactor biosynthesis [145, 146], including other steps of Moco biosynthesis [62], which
may be attributed to the small amount of cofactors required in the cells. Thus, my data presented here suggest that 3’,8-cH₂GTP may represent the physiologically relevant intermediate of Moco biosynthesis.

The MoaA reaction product has been described previously. Schindelin and Hänzelmann [71] first stated in their publication that in the reaction of MoaA with SAM and GTP without MoaC they observed the formation of small molecule that may be converted to DMPT. Unfortunately, no data were presented. My analysis in this report provides strong evidence that MoaA product indeed has an acid labile 6-hydroxy-2,4,5-triaminopyrimidine moiety that may be converted to DMPT. More recently, Begley et al. reported the observation of a molecule with a light absorption feature at 320 nm in the LCMS analysis of the reaction mixture of MoaA incubated with GTP, SAM and dithionite. The observation of a mass signal at $m/z = 524 \ [M+H]^+$ at the same retention time led the authors to propose this molecule as pyranopterin triphosphate (19, Figure 10d). While this conclusion is distinct from mine, the reported light absorption feature and the mass signal are also consistent with 3’,8-cH₂GTP. In addition, Begley et al. mentioned that the observed molecule was oxygen sensitive, which is also a characteristic of 3’,8-cH₂GTP. On the other hand, my NMR data are inconsistent with pyranopterin triphosphate [141]. Thus, I conclude that the previously described putative MoaA products [71, 75] were indeed 3’,8-cH₂GTP.
It is noteworthy that Begley et al. also considered 3’,8-cH₂GTP as an intermediate of cPMP biosynthesis, but suggested that it may only transiently exist during the MoaA catalyzed conversion of GTP to pyranopterin triphosphate [75]. This proposal appears to be based mostly on their observation of the deuterium transfer from the 3’ position of GTP to 5’-dA, and their proposal for the structure of the MoaA product as pyranopterin triphosphate, which I now believe to be a mis-assignment. While their deuterium tracer experiments support the formation of a radical at C-3’, prior to my work, to my knowledge, there was no precedent for the reaction between a C-3’ centered radical and C-8 in purine nucleosides or nucleotides. In addition, the radical formation at C-3’ could also be consistent with the previous proposals with 18 as an intermediate (Figure 10c) [74, 77] if the H-3’ abstraction was to take place after the formation of 18. There are precedents for formyl group transfer reactions by radical mechanisms [147]. My isolation and characterization of 3’,8-cH₂GTP as a MoaA product, provide a strong evidence for the function of MoaA to catalyze the conversion of GTP to 3’,8-cH₂GTP. As 3’,8-cH₂GTP can be chemically derivatized to DMPT, my model is also consistent with the observations by Schindelin and Hänzelmann [71].

My identification of 3’,8-cH₂GTP as the product of MoaA as well as the substrate of MoaC is a sharp contrast to the previous consensus in the field, where MoaA was considered largely responsible for the formation of the pyranopterin ring, and a relatively minor role was considered for MoaC [74, 75]. My observations suggest that
MoaC plays a major role in the formation of pyranopterin ring. The reactions catalyzed by MoaA and MoaC may be compared to the conversion of GTP into 7,8-dihydroneopterin triphosphate by GTP cyclohydrolase I in folate biosynthesis (see Figure S1a). The first step of the GTP cyclohydrolases I reaction is the hydrolysis of the imidazole moiety of the guanine base using a water activated on Zn$^{2+}$ in the active site [124, 148]. This hydrolysis triggers the ensuing complex Amadori rearrangement [120, 149-151] that takes place in the same active site [152, 153]. My results suggest that Moco biosynthesis is also initiated by modification of GTP at the C-8 position, but to a distinct molecule, 3’,8-cH₂GTP. In contrast to GTP cyclohydrolases I, MoaA is incapable of catalyzing the rearrangement reactions, presumably due to the lack of appropriate amino acid residues in the active-site. Instead, 3’,8-cH₂GTP is transferred to MoaC where a complex rearrangement reaction proceeds. In the following paragraphs, I will discuss the mechanisms of the reactions catalyzed by each of these two enzymes.

The observation of 3’,8-cH₂GTP formed from GTP by a radical SAM enzyme, MoaA, indicated to us that the reaction would proceed through a H-atom abstraction from the 3’ position. This is consistent with the deuterium tracer experiments by Begley et al. [75]. In reactions catalyzed by radical SAM enzymes, SAM may be used catalytically or stoichiometrically. However, the demonstration of the stoichiometry for radical SAM catalyzed reactions is frequently complicated by abortive cleavage of SAM, where SAM is reductively cleaved to methionine and 5’-dA regardless of product
formation. The abortive SAM cleavage has been reported for many radical SAM enzymes to various degrees [143, 154-158]. A notable example is the recent report on QueE by Bandarian et al. [154]. QueE catalyzes a complex rearrangement of 7-carboxy-7-deazaguanine to 6-carboxy-5,6,7,8-tetrahydropterin during deazapurine biosynthesis. Although SAM is used catalytically in this reaction, detectable amount of 5′-dA was formed and contained deuterium atoms that originated from substrate. Thus, although previous studies indicated a transfer of a deuterium atom at the 3′ position of GTP to 5′-dA [75], the lack of knowledge about the stoichiometry of the reaction left significant ambiguity about how SAM is used in the MoaA reaction. My observation of the stoichiometric formation of 5′-dA and cPMP in the MoaA/MoaC coupled assay, in combination with the demonstration of the stoichiometric transfer of a deuterium atom at the 3′ position of GTP to the methyl group of 5′-dA, is consistent with the stoichiometric consumption of SAM and H-atom abstraction from the 3′ position.
Figure 28: Proposed Mechanisms of MoaA and MoaC Involving 3’,8-cH₂GTP

(a) Proposed mechanism for the MoaA catalyzed conversion of GTP to 3’,8-cH₂GTP. (b) A model of the MoaA active site. SAM was modeled into the X-ray crystal structure of MoaA in complex with GTP[71] (PDB ID: 2FB3) based on the structural alignment with the X-ray crystal structure of MoaA in complex with SAM[12] (PDB ID: 1TV8). Oxygens are shown in red, nitrogens in blue, sulfurs in yellow, phosphorous in orange, and irons in brown. (c) Possible simplified mechanism of the MoaC catalyzed conversion of 3’,8-cH₂GTP to cPMP. P designates a phosphate group.

Based on the observations described above and my structural characterization of 3’,8-cH₂GTP, in combination with chemical precedents and the reported structures of MoaA, I propose a mechanism for the MoaA catalyzed reaction (Figure 28a). The deuterium tracer experiments of mine and Begley et al. [75] suggested that the reaction is initiated by the abstraction of H-3’ by 5’-dA•, generated by the reductive cleavage of SAM using the N-terminal 4Fe-4S cluster as a reductant. The resulting C-3’ radical (21) then attacks C-8 and generates an 3’,8-cycloGTP aminyl radical intermediate 22, which is
then converted to 3’,8-cH₂GTP. While the aminyl radical 22 could be reduced by a re-
abstraction of H-atom from 5’-dA, my observation of the stoichiometric formation of 5’-
dA in the MoaA/MoaC coupled assay relative to cPMP disfavors such mechanism. Thus,
another electron source is required. One possible reductant is the C-terminal [4Fe-4S]
cluster that binds GTP [71, 72]. A reduction of 5’,8-cyclo-deoxyadenosine aminyl radical
by reduced methylviologen ($E^\circ = -0.45$ V) [159] has been reported. Thus, considering the
reported redox potentials of related [4Fe-4S] clusters ($E^\circ = -0.4 \sim -0.6$ V) [160, 161], the C-
terminal [4Fe-4S] cluster may carry out the reduction of the aminyl radical. The redox
role of the C-terminal [4Fe-4S] cluster may be probed by EPR spectroscopy [162] and are
currently being investigated in my lab.

The reduction of the aminyl radical 22 may also be facilitated by the three
conserved Arg residues, R17, R266 and R268 (numbering based on S. aureus MoaA),
which are 3.2 – 4.6 Å from N-7 of GTP in the crystal structure [71]. Mutations in these
residues have been found in Moco-deficient patients [163]. MoaA variants with
mutations in these residues still bind GTP to a various degree, but do not produce cPMP
when assayed with MoaC [71]. These Arg residues may be assisting the aminyl radical
reduction by electrostatic stabilization of the anionic charge on the base, or by providing
a H⁺. Controlled movements of protons and electrons are often critical for redox
reactions in enzyme catalysis [164].
Another important feature of the active-site of MoaA is the presence of conserved hydrophobic amino acid residues, V167, I194 and I253, surrounding 2'-OH and 3'-OH of GTP (Figure 28b) [71]. No amino acid residues are in H-bonding interaction distance from 2'-OH and 3'-OH, which is inconsistent with the earlier proposal where MoaA catalyzes complex rearrangement reactions that require general acid/base catalysis at 3'-OH [75]. On the other hand, the structural feature of MoaA active site is consistent with my proposal for the function of MoaA to catalyze the conversion of GTP to 3',8-cH2GTP, in which no general acid/base catalysis is required.

The hydrophobic environment around 2'-OH and 3'-OH of GTP may also be important for directing the reaction of C-3' centered radical with C-8 by preventing side reactions. To my knowledge, the reaction of C-3' radical with C-8 of purine nucleoside/nucleotide is unprecedented [73, 125, 165]. Radical formation at C-3' in DNA and RNA results in a formation of 3'-keto-2'-deoxyribonucleotides or a C3'-C4' bond cleavage by complex mechanisms [165]. Reactions of the C-3' nucleotide radical in enzymes have been extensively studied for ribonucleotide reductase (RNR) [166, 167], where the radical formation on C-3' facilitates dissociation of 2'-OH. Mechanistic studies on RNR and related synthetic model systems suggest that the dissociation of 2'-OH requires general acid/base catalysts at 2'-OH and 3'-OH [167]. The difference in the active-site structures of MoaA and RNR that both generates a radical at C-3', but produce distinct products, may indicate the mechanism by which enzymes control
radical reactions. The roles of the MoaA active site amino acid residues are currently under investigation.

The following complex rearrangement reactions are catalyzed by MoaC. While MoaC was previously known to be essential for the biosynthesis of cPMP [10, 12, 80, 123], the lack of understanding about the structure of the substrate of MoaC precluded identification of the exact role of this enzyme during the conversion of GTP into cPMP. Thus, my finding that 3’,8-cH₂GTP may be the physiological substrate provides a means to study the mechanism of the complex rearrangement reaction catalyzed by MoaC.

The structures of MoaC [76-79, 168] or previous in vitro characterization of MoaC in the presence of MoaA [12] did not reveal any complex cofactor or metals in MoaC. Thus, the MoaC reaction appears to take place via general acid/base catalysis using amino acid side chain of MoaC. My characterization of 3’,8-cH₂GTP suggested susceptibility of this molecule to acid hydrolysis. Thus, the transformation of GTP into 3’,8-cH₂GTP by MoaA may be sufficient to make this molecule susceptible to complex rearrangement by MoaC presumably using general acid/base catalysts. While several different mechanisms may be possible, Figure 8 shows a possible mechanism for the conversion of 3’cH₂GTP to cPMP by MoaC most consistent with the currently available chemical and enzymological precedents. The reaction is likely initiated by hydrolysis of the constrained pyrrolidine and imidazoline rings of 3’cH₂GTP to an aldehyde 23, based on the susceptibility of 3’cH₂GTP to acid hydrolysis during
derivatization to DMPT (Figure 28c). Acid hydrolysis of various aminal molecules has been well documented [169]. The aldehyde 23 is then converted to a hexose 24. The mechanism of this reaction could be either a retroaldol-aldol rearrangement initiated by the deprotonation at 2’-OH, or an α-ketol rearrangement initiated by the deprotonation at 3’-OH. A similar rearrangement reaction has been reported for 1-deoxy-D-xylulose-5-phosphate reductoisomerase [170]. The hexose 24 is then converted to cPMP by formation of the pterin ring and the cyclic phosphate. Considering that MoaC binds nucleoside triphosphate stronger than di- and monophosphate [77], the cyclic phosphate formation may be the last or close to the last step of the catalysis. Studies to further narrow the possible mechanisms are described in subsequent chapters.

This work describes the isolation and characterization of 3’,8-cH₂GTP, an intermediate in Moco biosynthesis, which had previously eluded structural characterization. My finding defines the reactions catalyzed by MoaA and MoaC, and provides insights into their catalytic mechanisms. In contrast to the previous notion in the field, my results suggests a complex mechanism for the MoaC catalyzed reaction.

2.4. Materials & Methods

Materials

QAE A25 Sephadex resin, Guanosine 5’-triphosphate (GTP), [U-13C5,15N10]GTP, S-adenosyl-L-methionine (SAM), 5’-deoxyadenosine (5’-dA), dithiothreitol (DTT), sodium dithionite, 2,3-butanedione, and DMPT were purchased from Sigma-Aldrich. DEAE
sepharose FF resin was from GE Healthcare. [3-3H]Ribose was from Omicron Biochemicals Inc. Chemically competent E. coli DH5α and BL21(DE3) cells, and all PCR primers were from Invitrogen. pET expression plasmids were from Novagen. Calf-intestine alkaline phosphatase (CIAP, 20 U/mL) was from NEB. UV-vis absorption spectra were determined using a U-3900 UV-VIS ratio recording double-beam spectrometer (HITACHI) or Nanodrop 1000 (Thermo Scientific). Non-linear least square fitting of kinetic data was carried out using KaleidaGraph software (Synergy Software, Reading, PA). Anaerobic experiments were carried out in a UNIlab workstation glove box (MBaun, Stratham, NH) maintained at 10 ± 2 °C with O2 concentration < 0.1 ppm. All anaerobic solutions were degassed on a Schlenk line, and equilibrated in the glove box atmosphere for > 12 h. All plastic devices used for anaerobic use were evacuated for > 12 h in the glove box antechamber before bringing into the glove box. All DNA sequences were confirmed by Eton Bioscience Inc. PCR was carried out using PfuUltraII polymerase (Stratagene) according to the manufacturer’s protocol. All HPLC experiments were performed on a Hitachi L-2130 Pump equipped with an L-2455 diode array detector, an L-2485 fluorescence detector, an L-2200 autosampler and an ODS Hypersil C18 column (Thermo Scientific) housed in an L-2300 column oven maintained at 40 °C. Staphylococcus aureus MoaA and MoaC were expressed and purified by following published protocols[12] with minor modification as described in the Supporting Methods. The expression plasmids for SUF proteins[162] and MOCS1B[83]
were kindly provided by Dr. Herman Schindelin. The 5-methylthioribose kinase expression plasmid[171] was a generous gift from Dr. John Gerlt.

Cloning of *Staphylococcus aureus* MoaA and MoaC

*S. aureus* moaA and moaC genes were cloned by PCR from isolated *S. aureus* (Strain Newman) genomic DNA using a pair of primers [moaA-f (A CAT ATG GAG AAC AAA TAA AAG ATA AAC TAG G) / moaA-r (ATT ATC TCG AG TCC CTA CAC ATT AAC C)] or [moaC-f (GG AGG GGT CAT ATG ACT GAG TTT ACT C) / moaC-r (AAA AGC TCG AGA AAC CAG AAT TAA ATC ATC TG)]. PCR was carried out for 25 cycles with an annealing temperature of 58 °C. Adenine was then added to the 3′-terminus of the PCR product for TA ligation by incubation with GO-Taq Hot start polymerase (Promega) in the presence of 0.2 mM dATP at 72°C for 30 min. The product was ligated with pCR2.1-TOPO (Invitrogen) following the manufacturer’s protocol. After confirmation of the DNA sequence, the resulting plasmids were digested with NdeI and BamHI and the moaA and moaC genes were subcloned into the corresponding site of pET15b, yielding pET-HisMoaA and pET-HisMoaC, respectively.

Expression of MoaA, and MoaC.

MoaA was expressed based on published protocol[12] with minor modifications. *E. coli* BL21(DE3) cells were co-transformed with pET-HisMoaA and a plasmid for the expression of the SUF proteins (SufA, SufB, SufC, SufD, SufS and SufE)[162] and grown overnight on LB agar plates. All growths were carried out in the presence of
Chloramphenicol (25 mg/L) and Ampicillin (100 mg/L). A single colony was picked and grown in LB medium (5 mL) to saturation (~16 h). Two mL of this solution was diluted into 200 mL LB in a 500 mL baffled flask and incubated at 37°C until growth reached saturation. A portion of this culture (100 mL) was then used to inoculate 1.5 L of LB medium in a 2.8 L baffled flask and grown at 37 °C until an OD₆₀₀ reaches to 0.9-1.1, at which time the temperature was lowered to 30 °C and MoaA expression was induced with 0.5 mM IPTG. The culture was continued for additional 4-5 hours until saturation, and cells were harvested by centrifugation, frozen in liquid N₂ and stored at -80°C.

Typically, 2-3 g of wet cell paste/L were obtained. Expression of MoaC, and MOCS1B was performed identically using E. coli BL21(DE3) transformed with pET-HisMoaC or pET-MOCS1B, except that MoaC was expressed at 37 °C for 4 h, and MOCS1B was expressed at 15 °C for 20 h.

**Purification of MoaA**

MoaA was typically purified aerobically from 100-120 g of cell paste followed by anaerobic reconstitution of its 4Fe-4S clusters. All purification steps were carried out at 4°C. The cell paste was re-suspended in 5 volumes of buffer B (50 mM Tris-HCl pH 7.6, 0.3 M NaCl, and 10% (v/v) glycerol) supplemented with 5 mM β-mercaptoethanol (b-ME). The cells were lysed by two passages through a French pressure cell operating at 14 000 psi. After removal of cell debris by centrifugation (20000 g, 20 min, 4°C), the dark brown supernatant was incubated with Ni-NTA agarose resin (Qiagen, 100 mL in buffer
B) for 30 min with gentle rocking. The resin was then packed in a column (5 x 20 cm), and washed with buffer B supplemented with 20 mM imidazole and 10 mM b-ME, followed by elution with 250 mM imidazole. The fractions containing MoaA, as judged by the Bradford reagent and UV-vis spectroscopy, were then desalted using a Sephadex G-25 column (5 x 50 cm, 1 L) equilibrated with buffer B supplemented with 5 mM DTT. The resultant protein solution (~200 mM) was frozen in liquid N₂, and stored at -80°C.

The protein concentrations were determined based on UV absorption at 280 nm using the extinction coefficients (\(\varepsilon_{280\text{nm}} = 26.2 \text{ mM}^{-1} \text{ cm}^{-1}\)) determined by the Edelhoch’s method[172]. The amounts of Fe\(^{2+/3+}\) and S\(^2^-\) were quantified by following published protocol[173, 174]. Typically, 23 mg of MoaA with 2.3 Fe\(^{2+/3+}\) and 2.8 S\(^2^-\) per polypeptide were obtained per g of wet cell paste.

**Reconstitution of MoaA**

The as-isolated MoaA (200 µM) was degassed on the Schlenk line by three cycles of 3 s evacuation followed by 15 min of Ar refill. The sample was then brought into a glovebox, and equilibrated in the glovebox atmosphere for 1-2 h. Reconstitution was carried out at 10 °C by an addition of 9 eq. of Fe\(^{II}(NH_4)_2(SO_4)_2\) and Na₂S over the course of 30 min followed by incubation for an additional 30 min. The buffer was then exchanged into 50 mM Tris-HCl pH 7.6, by Sephadex G-25 column chromatography. The resultant protein solution (~100 µM) was frozen in liquid N₂, and stored at -80°C. The amounts of Fe\(^{2+/3+}\) and S\(^2^-\) were determined as described above for the as-isolated
MoaA purification. Since the UV absorption by the [4Fe-4S] clusters was significant at 280 nm (~20% of the protein absorption), the protein concentration was determined by the Bradford assay using the as-isolated MoaA as a standard. This reconstitution procedure reproducibly yielded MoaA with 8.5 ± 1.0 Fe$^{2+/3+}$ and 7.0 ± 0.5 S$^2$ per polypeptide and a specific activity of 2.17 ± 0.12 nmol/mg/min.

**Purification of MoaC**

A cell paste (16 g) of *E. coli* BL21(DE3) expressing MoaC was suspended in 70 ml of buffer A supplemented with 40 mM imidazole. The cells were lysed by two passages through a French pressure cell operating at 14,000 psi. After removal of cell debris by centrifugation (20,000 g, 20 min, 4 °C), DNA was precipitated by dropwise addition of 0.2 volumes of buffer A containing 6 % (w/v) streptomycin sulfate. The mixture was stirred for an additional 15 min, and the precipitated DNA was removed by centrifugation (20000g, 20 min, 4°C). The sample was then incubated with 10 ml Ni-NTA agarose resin (Qiagen) with gentle rocking. The resin was then packed into a column (1.5 x 10 cm) and washed with 4 volumes of buffer A supplemented with 40 mM imidazole, and MoaC was eluted with 250 mM imidazole in buffer A. Fractions containing MoaC were identified by SDS-PAGE, combined and exchanged into buffer A on a Sephadex G-25 column. Protein fractions were combined, concentrated by ultrafiltration (Amicon Ultra 3 K, Millipore) to 2 mM, flash frozen in liquid nitrogen and stored at -80 °C. The
concentration of MoaC was determined based on UV absorption at 280 nm using an extinction coefficient ($\varepsilon_{280\text{nm}} = 9.97 \text{ mM}^{-1} \text{ cm}^{-1}$) determined by the Edelhoch’s method[172].

**Stepwise Assay of MoaA and MoaC**

The stepwise MoaA/MoaC assays were carried out under anaerobic conditions. First, MoaA (5 µM) was incubated with GTP (1 mM), SAM (1 mM), and sodium dithionite (1 mM) in 200 mL of assay buffer (50 mM Tris-HCl pH 7.6, 1 mM MgCl$_2$, 2 mM DTT and 0.3 M NaCl). The reaction was initiated by the addition of MoaA, and incubated for 60 min at 25 °C, at which point MoaA was removed by ultrafiltration (Amicon Ultra 30 K, Millipore). To an aliquot (80 µL) of the resulting small molecule fraction, 10 mL of 50 mM MoaC was added and incubated for 60 minutes at 25 °C. The reaction was stopped by an addition of 10 µL of 25% (w/v) TCA. cPMP formed during the incubation with MoaC was oxidized to compound Z and quantified by HPLC following the published protocol[10]. Briefly, a solution (10 µL) containing 1% (w/v) I$_2$ and 2% (w/v) KI was added to the quenched reaction mixture, and incubated for 20 min at room temperature. After removal of precipitation by centrifugation, an aliquot (10 µL) of the supernatant was injected to HPLC. The chromatography was performed by an isocratic elution with 0.1% TFA in H$_2$O with a flow rate of 1 mL/min, and monitored by fluorescence (Ex. 367 nm, Em. 450 nm). Under these conditions, the void volume was 1.5 min and compound Z was eluted at 2.7 min. The authentic compound Z standard was isolated from $\Delta$MoeB *E. coli* strain by following the reported protocol[11].
Detection of the MoaA product as DMPT.

MoaA (5 µM) was incubated with GTP (1 mM), SAM (1 mM), and sodium dithionite (1 mM) in assay buffer (0.5 mL). The reaction was initiated by the addition of MoaA, and incubated at 25 °C for a specified time. An aliquot (80 µL) was removed at each time point and mixed with 10 µL of 0.5 M HCl to quench the reaction. The resulting mixture was incubated at 95 °C for 5 min to facilitate the hydrolysis, followed by an addition of 6 mL 1 M NaOH to adjust the pH to 8.5. The resulting solution was combined with 25 mL of 0.66% (v/v) 2,3-butanedione solution in 0.9 M Tris-HCl, pH 8.5, and incubated at 95 °C for 45 min. After removal of precipitation by centrifugation, an aliquot (10 µL) of the supernatant was injected to HPLC. The chromatography was performed by an isocratic elution with 92.5% 20 mM sodium acetate pH 6.0, 7.5% MeOH with a flow rate of 1 mL/min, and monitored by fluorescence (Ex. 365 nm, Em. 445 nm). Under these conditions, the void volume was 1.5 min and DMPT was eluted at 5.6 min.

3´,8-cH₂GTP Stability Test

The temperature and pH stabilities were assessed by incubating 3´,8-cH₂GTP (50 µM) solution in anaerobic buffer (15 mM ammonium formate pH 3.5, 50 mM Tris-HCl pH 7.6, or 15 mM ammonium bicarbonate pH 9.0) in the glovebox at 10 or 22 °C. To test for oxygen sensitivity, 3´,8-cH₂GTP (50 µM) solution was taken out of the glovebox, and exposed to air by rigorous pipetting. For each reaction, an aliquot (10 µL) was removed at specified time point and incubated with MoaC in 80 mL of assay buffer (50 mM Tris-
HCl, pH 7.6, 0.3 M NaCl, 1 mM MgCl\(_2\) and 2 mM DTT). The reaction was quenched with 10 µL 25% TCA, and cPMP was quantified as described above for the stepwise assay.

Isolation of 3´,8-cH\(_2\)GTP.

The MoaA reaction and subsequent purification of 3´,8-cH\(_2\)GTP were carried out under strict anaerobic conditions (< 0.1 ppm O\(_2\)). The large scale MoaA reaction for the isolation of 3´,8-cH\(_2\)GTP was carried out with MoaA (200 µM), SAM (1 mM), sodium dithionite (1 mM), and GTP or [U-\(^{13}\)C\(_5\),\(^{15}\)N\(_{10}\)]GTP (200 mM) in 50 mM Tris-HCl pH 7.6 (200 mL) at 22 °C for 60 minutes. These reactions typically produced 12-20 mmol of 3´,8-cH\(_2\)GTP. Subsequent purification was carried out at 10 °C. The MoaA reaction mixture was first passed through an ultrafiltration membrane (Amicon YM-30, Millipore) to remove the protein, and the resulting filtrate was applied to a QAE A25 Sephadex (250 mL, bicarbonate form) column. The column was washed with 750 mL of H\(_2\)O, and elution was performed by a linear gradient (600 x 600 mL) of 200 - 800 mM ammonium bicarbonate, pH 9.1. To identify the fractions containing 3´,8-cH\(_2\)GTP, an aliquot (10 µL) of each fraction was mixed with 80 mL of MoaC (20 µM) in assay buffer, and incubated for 30 min at 25 °C. The resulting solutions were analyzed for cPMP as described above for the stepwise assay. The fractions containing 3´,8-cH\(_2\)GTP were combined, lyophilized, re-dissolved in water and applied to a DEAE sepharose FF (15 mL, bicarbonate form) column. The column was washed with 50 mL of 100 mM ammonium bicarbonate.
bicarbonate, pH 9.1, and eluted by a linear gradient (300 x 300 mL) of 100 - 200 mM ammonium bicarbonate, pH 9.1. The fractions containing 3’,8-cH₂GTP were identified by UV-vis absorption spectroscopy and the MoaC activity assay described above. After removal of the solvent by lyophilization, the purified 3’,8-cH₂GTP was characterized by NMR and ESI-TOF-MS.

**Phosphate quantitation**

Phosphate quantitation was carried out based on the published protocol[11, 127]. Purified 3’,8-cH₂GTP (4 pmol) was incubated with CIP (5 units, each unit is defined as the amount of enzyme that hydrolyzes 1 nmol p-nitrophenylphosphate per 1 min at 37 °C) for 1 hour at 37 °C in 100 mL of 50 mM Tris-HCl pH 7.9 containing 100 mM NaCl, 10 mM MgCl₂ and 1 mM DTT. The resulting solution was then mixed with 0.233 mL of 1.4 % (w/v) ascorbic acid, 0.36 % (w/v) ammonium molybdate tetrahydrate in 0.86 M H₂SO₄. After an hour of incubation at 37 °C, the phosphate concentration was determined based on the absorption of the phosphomolybdate complex at 820 nm (ε₈₂₀ 𝑛𝑚 = 26.0 mM⁻¹ cm⁻¹)[127].

**Determination of the molecular weight of 3’,8-cH₂GTP**

Purified 3’,8-cH₂GTP (100 µM) in anaerobic water was prepared in the glovebox. An aliquot (10 µL) of the sample was injected into the ESI-TOF MS instrument (Agilent 6224) operated in the negative ion mode. The typical mass accuracy of the instrument was 5 ppm. For the characterization of the observed MS signal, 3’,8-cH₂GTP (100 µM)
was incubated with MoaC (50 μM) in 400 mL of 300 mM ammonium bicarbonate buffer pH 9.1 for 20 min at 25 °C. After removal of MoaC by ultrafiltration (Amicon YM-10, Millipore), the solution was lyophilized and the residue was dissolved in 400 mL of anaerobic water. The resulting solution was analyzed by ESI-TOF MS as described for 3’,8-cH₂GTP.

NMR measurements of purified 3’,8-cH₂GTP

All NMR spectra were recorded on 500, 600, or 800 MHz Varian Inova NMR spectrometers operated with VNMRJ 3.1 software, and analyzed by the ACD/NMR processor (ACD/Labs). The samples were dissolved in anaerobic deuterium oxide (Sigma-Aldrich, 99.9 atom% enriched), loaded in 3 mm NMR tubes (Wilmad LabGlass) in the glovebox, and sealed with butyl rubber septa (Sigma-Aldrich). NMR measurements were carried out for 24-48 h at a sample temperature of 6 °C. No decomposition of 3’,8-cH₂GTP was observed during the NMR measurements based on a comparison of ¹H NMR spectra at the beginning and the end of the measurements. Chemical shifts are reported in d based on an internal standard, 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS, dCH₃ = 0.00) as reference. Maleic acid (dCH = 6.0, Sigma-Aldrich) was used as an internal standard for signal quantitation.

MoaA/C coupled assay.

Enzyme activity assays were performed at 25 °C under anaerobic conditions by incubating MoaA (0.5 μM) and MoaC (5 μM) with GTP (1 mM), SAM (1 mM) and
sodium dithionite (1 mM) in assay buffer. The reaction was initiated by the addition of 
MoaA. At each time point, an aliquot (90 µL) was removed and mixed with 10 mL 25% 
(w/v) TCA to quench the reaction. cPMP was quantified as described above for the 
stepwise assay.

Preparation of MOCS1B expression plasmid

The MOCS1B gene in the pQE-MOCS1B plasmid originally prepared by 
Hänzelmann et al.[83] was subcloned into pET-28b to improve protein expression. NdeI 
restriction site was introduced by PCR at the 5’ end of the MOCS1B gene in the pQE-
MOCS1B plasmid using primers MOCS1B-f (CAT CAC CAT CAC CAT ATG ATG AGT 
TTC TC) and MOCS1B-r (GAG AAA CTC ATC ATA TGG TGA TGG TGA TG). After 
confirmation of the DNA sequence, the resulting plasmid was digested with NdeI and 
SalI and the MOCS1B gene fragment was subcloned into the corresponding site of 
pET28b to obtain pET-MOCS1B.

Expression of MOCS1B

E. coli BL21(DE3) cells were co-transformed with pET-MOCS1B and grown 
overnight on LB agar plates. All growths were carried out in the presence of Kanamycin 
(50 mg/L). A single colony was picked and grown in LB medium (5 mL) to saturation 
(~16 h). Two mL of this solution was diluted into 200 mL LB in a 500 mL baffled flask 
and incubated at 37 ºC until growth reached saturation. A portion of this culture (10 mL) 
was then used to inoculate 1.5 L of 2YT medium in a 2.8 L baffled flask and grown at 37
°C until an OD$_{600}$ reached 0.6-0.8, at which time the temperature was lowered to 15 °C and MOCS1B expression was induced with 0.3 mM IPTG. The culture was continued for additional 20 hours until saturation, and cells were harvested by centrifugation, frozen in liquid N$_2$ and stored at -80 °C. Typically, 10 g of wet cell paste/L were obtained.

**Purification of MOCS1B**

The cell paste (10 g) of *E. coli* BL21(DE3) expressing MOCS1B was suspended in 50 ml buffer A (50 mM Tris-HCl, pH 9.0, 0.3 M NaCl, 10% glycerol) supplemented with 1% Triton-X and a protease inhibitor cocktail (Calbiochem # 539132). The cell suspension was homogenized, and lysed by two passages through a French pressure cell operating at 14,000 psi. After removal of cell debris by centrifugation (20,000 g, 20 min, 4 °C), DNA was precipitated by dropwise addition of 0.2 volumes of buffer A containing 8% (w/v) streptomycin sulfate. The mixture was stirred for an additional 15 min, and the precipitated DNA was removed by centrifugation (20,000 g, 20 min, 4 °C). Solid (NH$_4$)$_2$SO$_4$ (0.23 g per mL) was then added over 15 min to 40% saturation. The solution was stirred for an additional 20 min and the precipitated protein was isolated by centrifugation (20,000 g, 20 min, 4 °C). The protein pellet was dissolved in a minimal volume of buffer A supplemented with 1% Triton-X, 0.5 mM phenylmethanesulfonylfluoride (PMSF) and 20 mM imidazole, and applied to a Ni-NTA column (10 ml). The column was then washed with 10 volumes of the same buffer, followed by another 10 volumes of buffer A containing 20 mM imidazole. MOCS1B was
subsequently eluted with 500 mM imidazole in buffer A. Fractions containing MOCS1B were identified by SDS-PAGE, combined and exchanged into buffer A on a Sephadex G-25 column. The resulting MOCS1B was concentrated by ultrafiltration (Amicon Ultra 3 K, Millipore) to 0.2 mM, flash frozen in liquid nitrogen and stored at -80 °C. The concentration of MOCS1B was determined based on UV absorption at 280 nm using an extinction coefficient (\( \varepsilon_{280\text{nm}} = 15.9\ \text{mM}^{-1}\ \text{cm}^{-1} \)) determined by the Edelhoch’s method [172].

**Activity Assays of MoaC and MOCS1B**

MoaC (0.1 µM) or MOCS1B (0.5 µM) was anaerobically incubated with 3’,8-CH₂GTP at specified concentrations in assay buffer at 25 °C. The reaction was initiated by the addition of MoaC or MOCS1B, and an aliquot (90 µL) was removed at each time point and mixed with 10 mL of 25% (w/v) TCA to quench the reaction. cPMP was quantified as described above for the stepwise assay.

**Quantitation of 5’-dA**

Enzyme reactions were performed and quenched as described for the MoaA/C coupled assay except that 20 µM MoaA and 40 µM MoaC were used. After the TCA quenching, the solution was clarified by centrifugation, and an aliquot (70 µL) of the supernatant was injected to HPLC. Chromatography was performed by a linear gradient of 0-15% MeOH in 27 mM KH₂PO₄ pH 4.5 (25 min, 2 mL/min), and monitored by UV absorption at 256 nm.

**Preparation of [3’-²H]GTP**
Enzymatic synthesis of [3´-2H]guanosine. [3-2H]Ribose (1 mM, > 95% enriched) was incubated at 27 ºC with ATP (2 mM), guanine (2 mM), 5-methylthioribose kinase[171] (3.5 mM) and purine nucleoside phosphorylase (Sigma-Aldrich, 3.5 mM) in 200 mL of 10 mM Tris-HCl pH 7.6 containing 2.5 mM MgCl₂ and 2.5 mM DTT. After 2 h of incubation at 27 ºC, the solution was adjusted to pH 3 with 1 M HCl, and loaded on to a DOWEX 50W-X8 column (2 x 10 cm, ammonium form). The column was washed with 15 mM ammonium formate pH 3.0, and eluted with 300 mM ammonium formate pH 9.0. Fractions containing guanosine were identified by UV-vis absorption spectrum and TLC. Guanosine was typically eluted after 7 - 10 CV of elution. The fractions containing guanosine were combined, and the solvent was removed by lyophilization to yield 100 mmol of [3´-2H]guanosine (94 ± 3 % atom ²H). ¹H-NMR (500 MHz, D₂O) δ 7.95 (s, 1 H), 5.86 (d, J = 7.3 Hz, 1 H), 4.62 (m, 1H), 4.18 (br, 1 H), 3.84 (dd, J = 2.9, 12.7 Hz, 1H), 3.77 (dd, J = 3.9, 12.7 Hz, 1H); HRMS (ESI-TOF) m/z [M+H]⁺ calcd. for C₁₀H₁₃DN₅O₅ 285.106; found 285.107.

Phosphorylation of [3´-2H]guanosine. Phosphorylation of guanosine was performed by the Ludwig’s method [175]. Briefly, [3´-2H]guanosine (100 µmol) was dried by co-evaporation with dry pyridine, and dissolved in dry (MeO)₃PO (1.5 mL). To this solution, POCl₃ (130 µmol) was added, and the mixture was stirred on ice for 2 h. Then, Bis-tri-n-butylammonium pyrophosphate (500 µmol) in anhydrous DMF (2 mL) and Bu₃N (100 mL) was added under rigorous stirring. After 10 min, 7 mL of 1 M
triethylammonium bicarbonate pH 7.5 was poured into the solution and stirred for 15 min. The resulting mixture was diluted by 5-fold with water, and applied to QAE A25 Sephadex (Sigma, 4 x 20 cm, bicarbonate form). The column was washed with 0.2 M ammonium bicarbonate pH 8.0 (100 mL), and the elution was performed with a linear gradient (450 x 450 mL) of 0.2 – 1 M ammonium bicarbonate pH 8.0. GTP was eluted between 0.55 – 0.7 M ammonium bicarbonate, judged by UV-vis absorption spectrum. Lyophilization of these fractions yielded 24 µmol of [3´-3H]GTP (94 ± 3 % atom 3H). 1H-NMR (500 MHz, D2O) δ 7.92 (s, 1 H), 5.71 (d, J = 5.9 Hz, 1 H), 4.56 (d, J = 5.9 Hz, 1 H), 4.15 (br, 1 H), 4.04 (m, 2 H); 31P-NMR (160 MHz, D2O) δ -8.04 (br), -11.26 (d, J = 19 Hz), -22.45 (br); HRMS (ESI-TOF) m/z [M-H] calcd. for C10H14DN5O14P5 522.990; found 522.991.

**MoaA reaction with [3´-3H]GTP and isolation of [5´-3H]5´-dA**

[3´-3H]GTP (98 µM) was anaerobically incubated with MoaA (50 µM), MoaC (100 µM), SAM (1 mM), and sodium dithionite (1 mM) in assay buffer for 1 hour at 25 °C. Proteins were removed by ultrafiltration (Amicon Ultra 30 K, Millipore), and the resulting filtrate was applied to a C18 RP Silica 90 column (Sigma, 2 x 8 cm) equilibrated in water. Elution was performed using a linear gradient (300 x 300 mL) with 0-30% MeOH in water. 5´-dA was eluted at 15% MeOH judged by UV-vis absorption at 256 nm. After removal of the solvent by rotary evaporator, the sample was further purified by HPLC using conditions identical to those described above for the quantitation of 5´-dA except that 30 mM ammonium formate pH 4.5 was used as the aqueous solvent. The
resulting sample was lyophilized and characterized by $^1$H NMR, and $^2$H NMR spectroscopies and ESI-TOF-MS.
3. Mechanism of Pyranopterin Ring Formation in Molybdenum Cofactor Biosynthesis

3.1. Introduction

While once again two enzymes, MoaA and MoaC, have been shown to be responsible for the formation of cPMP [10, 12, 123], their individual functions and catalytic mechanisms have been under significant debate. Previous studies have largely focused on MoaA because of the hypothesis that it catalyzes the majority of the rearrangement reaction by a mechanism involving free radical chemistry [12, 74, 75]. In line with such a view, MoaA was proposed to catalyze the conversion of GTP into pyranopterin triphosphate (Figure 29a) [75, 176]. However, the experimental demonstration of this model has been challenging due to the poor stability of the MoaA product especially in the presence of oxygen. Only recently, as described in Chapter 2, the MoaA product was isolated from experiments performed under strict anaerobic conditions [61]. NMR characterization suggested its structure as 3´,8-cH₂GTP (Figure 29b). This observation was in sharp contrast to the previous hypothesis, as it suggested that the pyranopterin ring may be constructed by MoaC, and not MoaA. MoaC does not show significant amino acid sequence homology to other functionally characterized enzymes, and no crystal structures were available in complex with its physiological ligands [76-79]. Therefore, how MoaC may catalyze such a complex rearrangement is largely unknown.
Here, I report the *in vitro* and *in vivo* functional characterization of MoaC and the first X-ray crystal structures of MoaC in complex with its highly air sensitive substrate and product. These studies provide strong evidence that 3',8-cH₂GTP is MoaC’s physiological substrate. Furthermore, the structures offer insights into the dynamic motions of the enzyme and the ligand (substrate/product). These combined studies provide strong support that MoaC catalyzes the conversion of 3',8-cH₂GTP into cPMP, and a detailed catalytic mechanism is presented.

**Figure 29: Previously Proposed Functions of MoaC**

(a) Previously proposed functions of MoaA and MoaC in that MoaA is assumed to form the pterin moiety for cPMP, generating a pyranopterin triphosphate product, which is then utilized by MoaC to form cPMP [75, 122]. (b) My proposal for cPMP formation occurs the MoaA product, 3',8-cH₂GTP, which was identified through the work outlined in the previous chapter.
3.2. Results

3.2.1. Identification of the MoaC Active Site

To elucidate the physiological function of MoaC, I took a multidisciplinary approach by combining the *in vivo* and *in vitro* functional characterization, and the visualization of the MoaC-substrate complex by X-ray crystallography. To this end, I first aimed to identify the location of MoaC active-site using Ala scanning of conserved amino acid residues. I hypothesized that general acid/base catalysis would be involved in the catalytic function of MoaC [61], and chose ten strictly conserved acidic and basic residues, identified from sequence alignments of MoaC homologs from all kingdoms of life (Figure 30). The selected amino acid residues were individually mutated to Ala (Figure 30, red asterisk). The resulting mutants were expressed, purified (>90% purity), and assessed for their catalytic functions using a coupled assay with MoaA, where the conversion of GTP into cPMP was monitored. These assays revealed six mutants with activities less than 1% of wt MoaC (Figure 31a). Notably, these six amino acid residues cluster in a conserved pocket in MoaC [78].
Figure 30: Amino Acid Sequence Alignment of MoaC

(b) MoaC Variant | $K_m$ (µM) | $k_{cat}$ (1/min)
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Wt (His$_6$-tagged)</td>
<td>0.21 ± 0.058</td>
<td>0.56 ± 0.027</td>
</tr>
<tr>
<td>Wt (non-tagged)</td>
<td>0.25 ± 0.040</td>
<td>0.53 ± 0.061</td>
</tr>
<tr>
<td>K51A</td>
<td>&lt; 1.0 $^a$</td>
<td>0.0010 ± 0.00024</td>
</tr>
<tr>
<td>H77A</td>
<td>1.6 ± 0.81</td>
<td>0.0023 ± 0.00019</td>
</tr>
<tr>
<td>E112A</td>
<td>2.4 ± 0.82</td>
<td>0.0031 ± 0.00043</td>
</tr>
<tr>
<td>E114A</td>
<td>4.3 ± 1.8</td>
<td>0.0021 ± 0.00031</td>
</tr>
<tr>
<td>D128A</td>
<td>&lt; 1.0 $^a$</td>
<td>0.00077 ± 0.00008</td>
</tr>
<tr>
<td>K131A</td>
<td>&lt; 1.0 $^a$</td>
<td>0.00070 ± 0.00014</td>
</tr>
</tbody>
</table>

(c) 3',8-cH$_2$GTP (µM) Rate (min$^{-1}$)  
(d) 3',8-cH$_2$GTP (µM) Rate (min$^{-1}$)  
(e) 3',8-cH$_2$GTP (µM) Rate (min$^{-1}$)  
(f) 3',8-cH$_2$GTP (µM) Rate (min$^{-1}$)
Figure 31: Steady-state Kinetic Characterization of Wt and Mutant MoaC

(a) In vitro activity of wt and mutant MoaC’s as determined by a coupled assay with MoaA. (b) Steady state kinetic parameters determined for the conversion of 3’,8-cH2GTP into cPMP. Only the upper limit (1 μM) was determined because the reaction rate became impractically low below this substrate concentration. The rates of the conversion of 3’,8-cH2GTP into cPMP were determined using (c) wt His6-MoaC (0.1 μM) and 3’,8-cH2GTP (0, 0.1, 0.5, 1.0, 5.0, 10, 15 μM); (d) wt-MoaC (non-tagged, 0.1 μM) and 3’,8-cH2GTP (0, 0.1, 0.5, 1.0, 2.0, 5.0 μM); (e) K51A, D128A, or K131A MoaC (1.0 μM) and 3’,8-cH2GTP (0, 1.0, 5.0, 10, 15 μM); and (f) H77A, E112A, or E114A MoaC (1.0 μM) and 3’,8-cH2GTP (0, 1.0, 2.0, 5.0, 10, 15 μM). Solid lines are non-linear fits to the Michaelis-Menten equation with kcat and Km values in Figure 1d. All data are averages of two or three replicates, and error bars are the standard deviations among the replicates.

The six MoaC mutants were further characterized by steady state kinetic analysis using 3’,8-cH2GTP (> 95% purity based on 1H NMR), anaerobically isolated from the MoaA reaction [61]. The wt MoaC exhibited cPMP formation with high affinity (Km = 0.21 – 0.25 μM). All six mutants exhibited diminished activity (< 0.1% Km/kcat) relative to wt MoaC, consistent with the coupled assay (Figure 31b). To demonstrate the physiological relevance of the in vitro characterizations, I performed functional complementation of a Moco-deficient E. coli strain lacking the wt moaC gene (E. coli ΔmoaC). Due to the essential role of Moco in nitrate reductase (NR) catalysis, the E. coli ΔmoaC strain does not express functional NR and exhibits significant growth defects under anaerobic nitrate respiration conditions[78, 177]. Genetic complementation of this strain with wt-MoaC rescued Moco biosynthesis as assessed by NR activity and bacterial growth under nitrate respiration conditions (Figure 32). On the other hand, complementation with mutant moaC genes exhibited poor growth under nitrate respiration conditions and very low or no detectable NR activity. These observations
indicate that MoaC residues K51, D128, and K131 are important for the \textit{in vivo} synthesis of Moco, supporting the physiological relevance of the \textit{in vitro} activity assays.
Figure 32: *In vivo* Complementation of Wt and mutant MoaC

(a) Moco production in *E. coli* ΔmoaC expressing wt or mutant MoaC. (b) Western blot detection of His$_6$-MoaC expressed in *E. coli* ΔmoaC. Overexposed Western blot (40 min) presented to show lower limit of detection of this method.
3.2.2. Structural Studies of MoaC in Complex with Substrate

To obtain structures of MoaC in complex with 3’8-cH₂GTP, the catalytically deficient K51A-MoaC was crystallized as an apo-form, and the crystals were incubated with 3’8-cH₂GTP. The resulting K51A-MoaC structure revealed a trimer of homodimers (Figure 33, 34: 1.78 Å resolution, $R_{\text{work}}/R_{\text{free}} = 17.38\%/19.35\%$, PDB ID 4PYA, Table 4) similar to that of the previously reported [78] wt apo MoaC structure. Unlike the apo structure, however, data from the soaked crystals revealed clear electron density in a pocket at the interface of two dimers and N-terminal end of helix α4 that was well fit with 3’8-cH₂GTP (Figure 33, 34). The presence of 3’8-cH₂GTP in the crystals was confirmed by dissolving the crystals in acid after data collection, followed by HPLC analysis (Figure 39). In the K51A-MoaC•3’8-cH₂GTP structure, the ligand forms extensive H-bonding interactions with catalytically essential and strictly conserved amino acid residues: The base moiety interacts with the main chain amides of H77, E112 and M113 and the side chain of E114; the aminal and ribose moieties contact the side chains of K131 and D128; and α-phosphate interacts with the side chain of H77. These multiple H-bonding interactions provide high specificity in 3’8-cH₂GTP binding by MoaC. Together with the in vivo and in vitro characterization of the wt and active-site mutants of MoaC, all support 3’8-cH₂GTP as the most likely physiological substrate of MoaC.
3.2.3. Structural Studies of MoaC in Complex with Product

3’,8-cH₂GTP and cPMP have distinct chemical structures, indicating that MoaC must perform a complicated rearrangement reaction to generate one from the other while retaining its binding to potential reaction intermediates whose structures are also likely different from these molecules. To obtain further insight into this mechanism, I solved the structure of wt MoaC in complex with cPMP by soaking experiments (Figure 35, PDB ID 4PYD). In this structure, density corresponding to cPMP was located in the same pocket as 3’,8-cH₂GTP at the N-terminus of the helix α4, providing further support that this pocket corresponds to the MoaC active site.
Table 3: Data Collection and Refinement Statistics for MoaC Crystallography

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<tr>
<th></th>
<th>MoaC-K51A•3',8-cH$_2$GTP (4PYA)</th>
<th>MoaC-cPMP (4PYD)</th>
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<tr>
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<td>38-3.18 (3.23-3.18)</td>
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<tr>
<td>$I/\sigma I$</td>
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<tr>
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<td>Ramachandran outliers (%)</td>
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</table>

*Values in parentheses are for highest-resolution shell.
Crystal structure of K51A-MoaC in complex with 3’,8-cH₂GTP revealed a trimer of homodimers. 3’,8-cH₂GTP (shown in sticks) was found at the N-terminus of helix a4 (highlighted in black).
The active-sites of K51A-MoaC in complex with 3',8-cH₂GTP. Simulated annealing omit maps for the ligands (mesh) were calculated with $F_o - F_c$ coefficients contoured at 3 $\sigma$. H-bond interactions are shown as indicated by dashed lines with the distances indicated.
Figure 35: Active Site of Wt-MoaC in complex with cPMP

The active-site of wt-MoaC with cPMP. Simulated annealing omit maps for the ligands (mesh) were calculated with $F_o - F_c$ coefficients contoured at 3 $\sigma$. H-bond interactions are shown as indicated by dashed lines with the distances indicated.
Figure 36: LigPlot of MoaC Structures with Ligands

LigPlot of (a) K51A-MoaC•3’,8-cH2GTP and (b) wt-MoaC•cPMP, and (c) overlay of both based on active-site residues.
Figure 37: Overlay of MoaC Structures

The overlay of the K51A-MoaC•3',8-cH2GTP (green) and wt-MoaC•cPMP (red) complex crystal structures.
Figure 38: Helix α4 in K51A-MoaC with 3’,8-cH₂GTP

Position of helix α4 relative to 3’,8-cH₂GTP in the K51A-MoaC•3’,8-cH₂GTP structure. Helix α4 is shown in magenta, and the dipole moment.
Figure 39: HPLC Analyses of 3′,8-cH₂GTP and cPMP in MoaC Crystals

(a) Derivatization scheme for 3′,8-cH₂GTP and cPMP to dimethylpterin (DMPT) and compound Z, respectively[61]. (b) HPLC quantitation of 3′,8-cH₂GTP and cPMP in MoaC crystals used for structure determination. Crystals were dissolved in 2.5% TCA after data collection to prevent any potential catalytic turnover. 3′,8-cH₂GTP and cPMP were then derivatized to dimethylpterin (DMPT) or compound Z, respectively, and quantified by HPLC as described in Methods. The presence of 3′,8-cH₂GTP in the crystals of K51A-MoaC, and the presence of cPMP in wt-MoaC were consistent with the electron density observed within the active site pocket.
3.2.4. Conformational Dynamics of the MoaC Structures

While the overall oligomeric structures of the substrate and product bound forms of MoaC are similar (Figure 37), significant structural differences were observed around the active-sites, specifically in the N-terminal loop and loop 3 (Figure 40a & b). In the K51A-MoaC•3’,8-cH₂GTP structure (Figure 40a), loop 3 points away from the active site and the N-terminal loop is disordered. By sharp contrast, in the wt-MoaC•cPMP complex (Figure 40b), loop 3 points towards the active-site, and the N-terminal loop folds and closes off the edge of the active-site. The closed conformation of loop 3 in the cPMP-bound structure is stabilized by the interaction between K51 and D128. This interaction is impossible in the substrate bound state due to the steric hinderance of 2’-OH of 3’,8-cH₂GTP (Figure 40a). The location of the 2’-OH of 3’,8-cH₂GTP in the substrate bound state overlaps with that of the K51 ε-amino group in the product bound state (Figure 40c), suggesting that loop 3 closure proceeds only after the conversion of 3’,8-cH₂GTP to a sterically less hindered intermediate. The observed N-terminal loop movement requires the ligand structural change because of its location in the cPMP-bound state overlaps with that of the triphosphate moiety of 3’,8-cH₂GTP. Specifically, in the substrate bound state, the α-phosphate of 3’,8-cH₂GTP is H-bonded with H77 (Figure 40a; also see Figure 34), whereas in the product bound state H77 is H-bonded to the backbone carbonyl of V16 (Figure 40b). The cyclic phosphate of cPMP is on the opposite side of the active-site, and H-bonded with E112, suggesting a large relocation
of the triphosphate moiety of the ligand during catalysis (Figure 40c). These findings indicate that the conversion of 3′,8-cH2GTP to cPMP by MoaC involves highly coordinated, large scale structural and conformational changes in the protein and ligand.

**Figure 40: Conformational Dynamics of MoaC and Ligands**

The crystal structures of the K51A-MoaC•3′,8-cH2GTP (a) and wt-MoaC•cPMP (b) complexes. Two subunits within a dimer are shown with different shades. H-bonding interactions between the N-terminal loop, loop 3, K51 and H77 are indicated by dashed lines with distances indicated in Å. (c) Overlay of the structures of K51A-MoaC•3′,8-cH2GTP (green), and wt-MoaC•cPMP (red). The conformational change of loop 3 and phosphate moiety of the ligands are indicated by arrows. The phosphate movement is likely coupled to irreversible cyclic phosphate formation as indicated by in a single headed arrow.

### 3.3. Discussion

The combined structural and enzymological studies suggest a likely mechanistic model for MoaC catalysis as shown in Figure 41. In this model, 3′,8-cH2GTP binds to MoaC with loop 3 in the open conformation. The first chemical step is likely the
hydrolysis of the aminal moiety (steps 1 and 2), based on the reported susceptibility of 3',8-cH²GTP to hydrolysis and the general chemical reactivity of aminal functional groups[61]. This reaction is facilitated by anion stabilization at O-6 through H-bonding interactions between O-6 and the amide NH of E112 and M113. This effect may be enhanced by the dipole moment of helix α4 (Figure 38) [178]. In the next step, a ring expansion proceeds by a retroaldol-aldol type rearrangement using D128 and K131 as a general acid/base catalyst (steps 3 and 4). The chemical conversion up to this point alters the structure of substrate significantly, and relaxes the sugar conformation, which may allow loop 3 closure and incorporation of K51 in the active site (step 5). K51 could then act as a general acid catalyst to assist in 2’-OH dissociation. Loop 3 closure will also stabilize folding of the N-terminal loop at the side of the active site (step 6), which accompanies the relocation of the triphosphate moiety and facilitates cyclic phosphate formation (step 7). Therefore, the model suggests the importance of the protein conformational flexibility, which allows for the precise coordination of substrate and protein structural changes in the MoaC-catalyzed rearrangement reaction.
Figure 41: Current Mechanism of MoaC-catalyzed 3’,8-ch2GTP conversion into cPMP

My currently proposed mechanism of the MoaC-catalyzed 3’,8-ch2GTP conversion into cPMP, based on new structural data. Shown are reaction intermediates, key amino acid residues and their H-bonding or electrostatic interactions (dashed lines).

Prior to this work described here, MoaA was suggested to catalyze the conversion of GTP into pyranopterin triphosphate (Figure 42a) [75, 176]. However, my recent work with carefully isolated and characterization MoaA produced suggested its structure as 3’,8-ch2GTP (Figure 42b). This observation was in sharp contrast to the previous hypothesis, as it suggested that the pyranopterin ring may be constructed by MoaC, and not MoaA. The ligand-bound MoaC structural studies described above have provided us with further support of my model. Using a re-refined omit map for K51A-MoaA without ligand (calculated after ligand removal and performing 50 rounds of refinement), I docked and compared the fit of two previously proposed MoaC
substrates, 3’,8-cH2GTP and pyranopterin triphosphate (Figure 42c). As it evident from this panel, only 3’,8-cH2GTP fits the density. Specifically, while 3’,8-cH2GTP and pyranopterin triphosphate both share the aminopyrimidinone moiety (which is tightly anchored by specific H-bonds from the Glu114 side chain to the guanine N1 and N2 atoms) the remainder of the electron density provides an optimal fit to that of 3’,8-cH2GTP, while it does not accommodate the third ring of the pyranopterin triphosphate at all (Figure 42d & e). Moreover, as the pyranopterin triphosphate rings buckle, they do not conform to the flat density as can be observed in Figure 42f. When I try rotating the pyranopterin triphosphate to accommodate the third ring, the aminopyrimidinone moiety is flipped and does not dock into the density. Additionally, in this position the Glu114 contacts to the aminopyrimidinone group are not only abrogated, but the aminopyrimidinone O6 atom now clashes with the Glu114 side chain Oε1 (Figure 42g).

The density for the triphosphate group is weaker (Figure 42c). This moiety makes few contacts to the protein and does not fit the density as well as the ring structure. However, as explained below, these findings are actually consistent and corroborative with my model, which involves dynamic movement of the ligand coupled with the phosphate reaction (see Figures 40 & 41). Hence, the high-resolution structure provides an extremely clear delineation between the two models, in favor of 3’,8-cH2GTP functioning as the MoaC substrate (product of MoaA).
Figure 42: Validation of MoaC Substrate Using High-Resolution Structural Data

(a) Previously proposed MoaA product, pyanopterin triphosphate [75, 122]. (b) The MoaA product, 3’,8-cH₂GTP, which was identified through the work outlined in the previous chapter. (c) – (g) Two proposed substrates of MoaC modeled into a re-refined omit map for K51A-MoaA calculated after ligand removal and performing 50 rounds of refinement.
In addition, I previously completed NMR characterization of the MoaA reaction (Chapter 2, Figure 17c), where the MoaA reaction was directly monitored by $^{13}$C NMR without any additional workup. This experiment provides evidence that 3’,8-cH₂GTP is the sole product of MoaA. In this experiment, the MoaA activity assay was performed using $^{13}$C-labeled GTP in an anaerobically sealed NMR tube. The resulting sample was directly subjected to $^{13}$C NMR analysis. Since this experiment does not require purification or chemical derivatization, the result represents the physiologically relevant MoaA product. As a consequence, the observed NMR spectrum (Chapter 2, Figure 17c red trace) consists of the signals from GTP and 3’,8-cH₂GTP (Chapter 2, Figure 17c, green and blue traces, respectively). Considering that the reported $^{13}$C NMR spectra of pyranopterin molecules have characteristic signals at 90-91 ppm, the observed $^{13}$C NMR data are inconsistent with pyranopterin triphosphate as the product of MoaA. These observations provide strong support for 3’,8-cH₂GTP as the sole product of MoaA.

In conclusion, my in vivo and in vitro functional characterization, and X-ray crystallography characterization of MoaC provide strong evidence that 3’,8-cH₂GTP is most likely the physiological substrate of MoaC. The crystal structures also indicate the importance of dynamic conformational changes within the MoaC active site during the catalysis. Considering the strict conservation of the MoaC catalytic residues identified in this study, the function and mechanism of MoaC homologs is likely conserved among all kingdoms of life, from bacteria to humans.
3.4. **Materials & Methods**

**General Materials**

Guanosine 5’-triphosphate (GTP), S-adenosyl-L-methionine (SAM), dithiothreitol (DTT), sodium dithionite, sulfanilamide, and 8-anilino-1-naphtalene sulfonic acid were purchased from Sigma-Aldrich. ΔmoaA, ΔmoeB, ΔmoaC E. coli strains were obtained from the KEIO KO collection[179]. Non-linear least square fitting of kinetic data was carried out using KaleidaGraph software (Synergy Software, Reading, PA). Anaerobic experiments were carried out in an UNIlab workstation glove box (MBaun, Stratham, NH) maintained at 10 ± 2 °C with an O₂ concentration < 0.1 ppm. All HPLC experiments were performed on a Hitachi L-2130 Pump equipped with an L-2455 diode array detector, an L-2485 fluorescence detector, an L-2200 autosampler and an ODS Hypersil C18 column (Thermo Scientific) housed in an L-2300 column oven maintained at 40 °C.

**Expression and purification of Escherichia coli MoaC**

The untagged wt E. coli MoaC was homologously expressed in E. coli C41(DE3) harboring a pMW15aC plasmid, and purified using a phenyl-sepharose column (20 mL, HiTrap 16/10 Phenyl FF, GE), as previously described.[78] For the expression of MoaC with a hexahistidine (His₆) tag at the N-terminus, the pMW15aC plasmid was digested with NcoI and BamHI and the moaC gene was subcloned into the corresponding site of pET30b (Novagen) to give pET-HisEcMoaC. The resulting plasmid was introduced into E. coli BL21(DE3), and His₆-tagged MoaC was expressed and purified as previously
described.[61] MoaC variants with mutations in one of the conserved amino acid residues (D17A, K21A, R26A, K51A, H77A, E112A, E114A, D128A, K131A, K147A) were prepared by following the Stratagene’s QuikChange site-directed mutagenesis protocol using the primers shown in Table S3 and the pET-HisEcMoaC or pMW15aC plasmid as a template.

Table 4: DNA Primers Used for Site-Directed Mutagenesis of MoaC.

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<th>Sequence</th>
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<tr>
<td>D17A-r</td>
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</table>

In vitro characterization of MoaC

For the coupled assay, purified wt- or mutant MoaC (0.5 µM) was anaerobically incubated with MoaA (1 µM, prepared as previously reported[61]), GTP (1 mM), SAM (1 mM), and sodium dithionite (1 mM) in the assay buffer (50 mM Tris-HCl pH 7.6, 1 mM MgCl₂, 2 mM DTT and 0.3 M NaCl) at 25 °C for 60 min. The reaction was then quenched
with 0.1 volume of 25% trichloroacetic acid (TCA), and cPMP was quantified by HPLC after its conversion to a fluorescent derivative, compound Z, as previously reported.[61] For the steady state kinetic analysis, 3’-8-CH₂GTP was isolated from the *in vitro* assay solution of MoaA as previously reported under strict anaerobic conditions (< 0.1 ppm O₂)[61]. Based on NMR and HPLC, the purity of 3’,8-CH₂GTP used in this study was greater than 95%. MoaC (0.1 or 1 µM) was anaerobically incubated with purified 3’,8-CH₂GTP at specified concentrations in assay buffer at 25 °C. The reaction was initiated by the addition of MoaC, and an aliquot (90 µL) was removed at each time point and mixed with 10 µL of 25% (w/v) TCA to quench the reaction. cPMP was quantified by HPLC as described above.

**In vivo characterization of MoaC**

For the complementation of *E. coli* ΔmoaC with MoaC variants, the pET-HisEcMoaC plasmid was digested with XbaI and HindIII and the moaC gene (wt or a mutant) was subcloned into the corresponding site of pBAD33 (ATCC) to give pBAD33-HisEcMoaC. To test the activity of MoaC mutants *in vivo*, the pBAD33-HisEcMoaC plasmid harboring one of the MoaC mutants was introduced into the *E. coli* ΔmoaC strain. The resulting transformants (*E. coli* ΔmoaC/pBAD33-HisEcMoaC) were cultured for 16 hr at 37 °C in LB media supplemented with kanamycin (50 mg/L) and chloramphenicol (34 mg/L). The cells from a 0.5 mL aliquot of these overnight cultures were pelleted and the media was removed. Cell pellets were brought inside an anaerobic
glove box (< 1 ppm O₂) and resuspended in 15 mL of anaerobic PN medium[39] supplemented with NaNO₃ (100 mM), kanamycin (50 mg/L), chloramphenicol (34 mg/L), 0.4% (v/v) glucose, and 1x trace metals[180]. Cultures were grown over 24 hr at 22 °C while shaking and the OD₆₀₀nm monitored. To determine the NR activity, the cells after 24 hr were harvested by centrifugation (5,000 g, 20 min, 4 °C), resuspended in 1 mL of 250 mM NaH₂PO₄ pH 7.1, and lysed at 4 °C by sonication using a Branson Digital Sonifier at 30% amplitude for 30 s with 0.5 s pulses. The protein concentration of the cell lysate was determined by the Bradford assay (Amresco). NR assays were performed at 22 °C using the whole cell lysate (100 µg/mL), 52.4 mM NaNO₃, 1.3 mM DTT, 0.204 µM methyl viologen, 2.2 µM sodium dithionite in 250 mM NaH₂PO₄ pH 7.1 (500 µL). The reaction was quenched by vigorous mixing with air, and nitrite was quantified by the diazocoupling derivatization method as previously described.[181]

Western Blot

Basal expression levels of His₆-MoaC from the uninduced pBAD33-HisEcMoaC complemented ΔmoaC E. coli growths were assessed by Western Blotting. SDS-PAGE was performed under reducing conditions on whole cell lysate using 12.5% polyacrylamide gels with 1-10 µg of total cellular protein per lane. After electrophoresis, proteins were transferred to a nitrocellulose membrane at 2.5 A for 20 min. His₆-MoaC was detected using anti-polyhistidine tag mAbs (Thermo Scientific) and peroxidase-coupled anti-mouse Ig (Southern Biotech). The observed bands were quantified based on
comparison with bands of purified His-MoaC standards (0, 0.34, 1.0, 3.1 pmol/lane). The concentrations of MoaC in *E.coli* cells were calculated based on the cell counting (1 x 10⁹ cells/mL/OD₆₀₀nm), and the reported volume of an *E. coli* cell as 0.85 fL[182].

**Crystallography and Structure Determination**

*E. coli* MoaC was crystallized by hanging drop vapor diffusion using a 1:1 ratio of 10 mg/mL MoaC to 35% PEG 1500 and 0.1 M MMT buffer [1:2:2 DL-Malic acid:MES(N-morpholino)ethane-sulfonic acid] at pH 9.0. Hexagonal bipyramidal crystals grew after 4 days at 22 °C. For soaking experiments, the apo crystals were transferred to a 3 µL drop of the crystallization solution on a coverslip in an anaerobic environment (< 0.1 ppm O₂) at 10 °C, and left to exchange for 1 hr with either 3’,8-cH₂GTP or cPMP at final concentrations of 0.27 mM and 0.83 mM, respectively. The coverslip was then sealed over an empty well for 15 hr, which led to partial dehydration of the crystals and improved diffraction. For data collection, the crystals were cryopreserved using the crystallization solution supplemented with 22% ethylene glycol. X-ray intensity data were collected on a R-AXIS HTC imaging plate area detector mounted on a Rigaku FRE+ SuperBright rotating anode generator using Cu Kα radiation, or at the SERCAT (South Eastern Regional Collaborative Access Team) 22-ID line at the Advanced Photon Source (Argonne National Laboratory). The data were processed with HKL3000[183]. All crystals took the hexagonal space group P6₃22 except the cPMP soaked crystals in which soaking resulted in an alteration of the symmetry to
the orthorhombic space group P2\(_1\)2\(_1\)2\(_1\). The structures were solved by molecular replacement using the apo *E. coli* MoaC structures as a search model (PDBID: 1EKR) with PHASER[184]. Subsequent model building was carried out using COOT[185] and refinement was performed in PHENIX[186]. Confirmation of a bound ligand was determined by dissolving co-crystals after data collection and analyzing by HPLC for 3',8-cH\(_2\)GTP or cPMP as described previously[61]. Coordinates and structure factors for the K51A-MoaC•3',8-cH\(_2\)GTP, and wt-MoaC•cPMP structures have been deposited in the Protein Data Bank under the accession codes 4PYA and 4PYD, respectively.
4. Protein C-terminus Assisted Radical Initiation By The Radical SAM GTP Cyclase, MoaA, in Molybdenum Cofactor Biosynthesis.

4.1. Introduction

More than 50% of the identified mutations found in Moco deficiency patients occur in MOCS1A, the human homolog to MoaA [61, 75, 105, 187]. MOCS1A, like MoaA, is essential for the formation of the pyranopterin backbone structure of Moco, and belongs to the radical SAM (S-adenosyl-L-methionine) superfamily (Figure 43a) [11, 12]. Enzymes in this superfamily catalyze the reductive cleavage of SAM using oxygen sensitive 4Fe-4S clusters, and transiently generate a 5´-deoxyadenosyl radical (5´-dA•), which then abstracts a H-atom from the substrate to initiate radical reactions [188]. MOCS1A has a second 4Fe-4S cluster that binds its substrate, GTP (Figure 43e) [12]. Although the product of MOCS1A is currently under significant debate (Figure 43b) [61, 142], evidence suggests that the enzyme catalyzes an H-atom abstraction from the 3´-position of GTP using 5´-deoxyadenosyl radical (5´-dA•) generated by a reductive cleavage of SAM (Figure 43c) [61, 75].

Of the mutations frequently found in MOCS1A that result in MoCD, a large majority of these are located in three locations (hot spots). Two of the hot spots, Cys ligands of the two 4Fe-4S clusters, and the conserved cationic residues, are located in the active-site, based on the X-ray crystal structures of bacterial homolog, MoaA (Figure 43d, 43e) [162]. On the other hand, the third hot spot, two consecutive Gly at the C-
terminus (GG motif), was disordered in the reported X-ray crystal structures, and significant ambiguity remains about their roles and the mechanism by which their mutations cause MoCD. The GG motif is conserved in all MOCS1A/MoaA homologs in prokaryotes and eukaryotes (G339 and G340 in S. aureus, Figure 44). The significance of the GG motif for the function of MOCS1A was shown by heterologous expression of MOCS1A in E. coli lacking moaA gene [83]. While wt MOCS1A complemented the Moco biosynthesis in E. coli ΔmoaA strain, MOCS1A with mutations in the GG motif did not. While these studies demonstrated the importance of the GG motif, the function of the motif and the mechanism by which its mutations cause MoCD have remained elusive.
Figure 43: Moco Biosynthesis and the Disordered C-terminal GG Motif

(a) Moco biosynthetic pathway in bacteria and humans. The first step of Moco biosynthesis is the formation of cyclic pyranopterin monophosphate (cPMP, 2) from the nucleotide, guanosine triphosphate (GTP, 1) by the enzymes MoaA (MOCS1A) and MoaC (MOCS1B). The human enzymes are indicated in parenthesis. (b) Previously proposed functions of MoaA and MoaC. Begley et. al. suggests that MoaA forms the pterin moiety for cPMP, generating a pyranopterin triphosphate product (19), which is then utilized by MoaC to form cPMP [75, 122]. I propose that cPMP formation occurs the MoaA product, 3’,8-cH$_2$GTP, which was identified through my previous work [61]. (c) Proposed mechanism of MoaA. (d) S. aureus MoaA homodimer in complex with SAM [12]. Disordered C-terminal tail drawn in green, last ordered residue, Q329, highlighted in blue, and the acidic residues investigated for the C-terminal tail binding are highlighted in red. (e) Model of S. aureus MoaA active site created by overlaying the structures of MoaA in complex with GTP, and MoaA in complex with SAM [12]. Illustrated are the active-site residues frequently found mutated in MoCD patients (hot-spots). These include the active site arginines and cysteines ligating the two 4Fe-4S clusters, shown in yellow.
C-terminal GG motifs have been found in ubiquitin and ubiquitin-like (Ubl) proteins. In these enzymes, the GG motif binds to the active-site of partner enzymes that posttranslationally modified the C-terminus of the Ubl proteins with sulfide or ubiquitine ligase [189]. However, MoaA/MOCS1A catalysis does not require sulfuration or ubiquitination, and therefore, novel function for the GG motif was expected. To elucidate the role of the GG motif, I used S. aureus MoaA, and characterized the function of GG motif by a combination of site-directed mutagenesis and the peptide rescue of the mutations. My work has demonstrated that synthetic 11-mer peptides corresponding to the C-terminal amino acid sequence of wt MoaA effectively rescue the catalytic activity of MoaA variants with mutations in the GG motif. Further biochemical and functional characterization suggested that the GG motif is critical for the binding of SAM and affects the H-atom transfer step. The C-terminal tail interaction site was probed by peptide rescue of MoaA variants with mutations in the putative site of interaction, which provided strong evidence for the interaction of the tail with the SAM-binding pocket of MoaA. Based on these observations, I propose that the GG motif interacts with the active-site of MoaA, and provides an essential mechanism in the control of the formation of the highly reactive 5’-dA•. This study provides another example for GG motif being essential in the interaction with an enzyme active-site. However, in the case of MoaA, the interaction is essential not for the posttranslational modification, but for the initiation of a radical reaction.
4.2. Results

4.2.1. Functional Characterization of MoaA GG Motif

While Ubl proteins with C-terminal GG motifs are posttranslationally modified at the C-terminus [191]. Therefore, I first investigated for any evidence of post-translational modifications (PTMs) in isolated MoaA. To this end, I used *S. aureus* MoaA heterologously expressed in *E. coli* and purified to > 95% homogeneity [61]. The purified MoaA was catalytically active in production of cPMP from GTP in the presence of the second enzyme, MoaC (bacterial MOCS1B homolog). The purified MoaA was treated with trypsin and analyzed by LC-MS (Figure 45). The analysis revealed only the unmodified, digested, C-terminal fragment (INMNYIGG, [M+H]^+ = 881.419), and no previously reported PTMs, such as sulfuration or amidation, were observed. While transient modification of the GG motif during the catalytic cycle cannot be ruled out, the observation suggests that the unmodified GG motif is catalytically competent.

To further investigate the function of GG motif, I prepared MoaA variants with mutations in the GG motif at either the conserved G339 or G340. Variants tested included single point mutations of Gly to Ala, Ser, Val, or 5 or 11 amino acid truncations.
at the C-terminus producing Δ336-340 and Δ330-340 MoaA, respectively. All mutants were heterologously expressed in *E. coli* similar to the wild-type enzyme and subsequently purified and anaerobically reconstituted to give holo-proteins with > 90% purity (Figure 46). All of these mutants were reconstituted with an average of 1.7 ± 0.28 4Fe-4S clusters per monomer, comparable to that found in wt-MoaA (2.1 ± 0.03 4Fe-4S clusters per monomer), suggesting that the GG mutations to the C-terminus of MoaA have little effect on the cluster loading. Similarly, using anaerobic size exclusion chromatography in the presences of SAM and GTP, the single point mutants migrated identical to that of wt MoaA indicating that the GG mutations are also likely not affecting the overall oligomeric state of MoaA (Figure 46). However, despite these observations, none of the GG motif mutants were catalytically active judged based on the formation of the MoaA product, 3’-8-cH₂GTP (Figure 47a). The results suggest that even a minimal perturbation to the GG motif, such as an introduction of a methyl group, completely abolishes the catalytic function of MoaA without significantly affecting the cofactor loading or the overall structure of MoaA.
(a) Total Ion Chromatography from positive-ion LC-MS Analysis of 50 µM Trypsin-Digested Wt-MoaA and 50 µM 8-mer C-terminal Peptide Std (INMNYIGG, [M+H]+ = 881.420). Unmodified C-terminal tail indicated with arrow. (b) Analysis of the digested wt MoaA indicated only the unmodified C-terminal fragment, [M+H]+ = 881.419. No common GG PTMs, such as 5'-adenosylation (+329 m/z) or Gly amidation (-58 m/z), were detected in the digested peptide fragments.
4.2.2. Peptide Rescue of MoaA GG Mutant Activity

To further investigate the functions of the GG motif, I sought for a rescue of the MoaA catalytic activity by peptides that correspond to the C-terminal amino acid sequence. This approach is based on my hypothesis that the C-terminal tail of MoaA is conformationally flexible and its dynamic interaction with the remaining parts of MoaA is essential for the catalysis. In the reported crystal structures of MoaA, the C-terminal 11
amino acids (RKKINMNYIGG\textsuperscript{340}), including the GG motif, are disordered (Figure 43d). Therefore, I prepared synthetic peptides corresponding to the disordered region of the C-terminus of MoaA and tested for their ability to restore the catalytic function. In the presence of an 11-mer peptide (RKKINMNYIGG), the GG motif mutants exhibited catalytic activity comparable to that of wt-MoaA (Figure 47a, “11-mer Wt Peptide”). The observed activity was dependent on the concentration of the peptide, based on which the $K_d$ value for the interaction was determined as $0.15 \pm 0.04$ mM (Figure 47b). Apparent cooperatively in the binding of the peptide was observed (Hill Coefficient = $2.0 \pm 0.2$), and a maximum activity ($k_{\text{cat}} = 0.054 \pm 0.005$ min\textsuperscript{-1}), comparable to that of the wt MoaA (Table 1, $k_{\text{cat}} = 0.042 \sim 0.045$ min\textsuperscript{-1}). An 11-mer peptide with a mutation in the GG motif (RKKINMNYIGA) failed to restore the MoaA activity even at 1.0 mM peptide concentration, suggesting the critical role of the GG motif in the peptide. Shorter peptides, 8-mer (INMNYIGG) and 5-mer (NYIGG), were also capable of activity restoration, but at significantly slower rate. The maximum activity observed for the 8-mer and 5-mer peptides were $\sim10$ and $\sim100$ times less than that by the 11-mer peptide, respectively, when tested at 200 mM peptide concentrations (Figure 47c). These results demonstrate the importance of the amino acid sequence and/or peptide length for the restoration of the MoaA mutant activity. All together, these observations suggest that a peptide corresponding to the disordered region of the C-terminus of wt MoaA can effectively rescue the activity of GG motif mutants \textit{in vitro} with certain specificity.
Figure 47: Characterization and Peptide Rescue of MoaA GG Mutant Activity.

(a) Activity of MoaA GG-motif mutants in the presence of the 11-mer peptide with amino acid sequence corresponding to the wt-MoaA or G340A-MoaA. The $k_{cat}$ values were determined based on formation of cPMP in the MoaA (3 µM) assay solution containing 10 µM MoaC, 0.3 mM NaCl, 1 mM SAM, 1 mM DTH, 5 mM DTT, 2 mM MgCl₂, 1 mM GTP, and 500 µM of specified peptide. Reactions were triplicated, and error bars represent standard deviation. (b) Activity of G340A-MoaA in the presence of various concentrations of the 11-mer Wt peptide. The solid line is the non-linear fit to Hill equation [192] with $k_{cat} = 0.055$ min⁻¹, $K_o = 151$ µM, and Hill coefficient = 1.86. (c) Activity of MoaA variants with mutations in the anionic and cationic residues. The activity assays were performed as in (a) using 200 µM of specified peptide.
4.2.3. The Role of MoaA GG Motif in SAM Binding

To obtain further insights into the role of the MoaA GG motif, I used the peptide rescue assay to characterized five GG motif mutants (four single mutants at 339 or 340 position, and one D330-340 truncated mutant) (Table 5). The steady state kinetic analysis was performed either without peptide, or with the 11-mer peptide at concentrations close to its $K_d$ or at its saturation (100 or 500 µM). In all tested mutants, in the presence of the saturating concentration of peptides, $k_{cat}$ values comparable to that for wt-MoaA were observed ($0.021 - 0.055$ min$^{-1}$ for mutants and $0.045 \pm 0.007$ min$^{-1}$ for wt-MoaA).

This observation shows the ability of the 11-mer peptide to rescue the activity of MoaA with any GG motif mutation.

The mechanistically most important observation was the perturbations in the $K_m$ and $K_d$ for SAM with minimal perturbation in those for GTP. In all the mutants tested, the $K_m$ values for SAM in the presence of saturating concentration of the peptide were 7~18-folds higher than that for wt-MoaA. In the anaerobic isothermal titration calorimetry (ITC) experiments, no thermal binding event was observed when SAM was titrated to mutant MoaA’s ($K_d > 90$ µM, Table 1, Figure 48), whereas clear exothermic binding events observed for wt-MoaA with $K_d = 1.7 \pm 0.6$ µM (Table 1, Figure 48). These observations were in sharp contrast to those for GTP, where the change in the $K_m$ and $K_d$ values were minimal ($K_m$, 2~6-folds increase from wt-MoaA; $K_d = 0.5$-5.2 µM in mutants vs 5.0 µM in wt MoaA). The observed perturbations in $K_m$ and $K_d$ for SAM binding, and
not for GTP binding, therefore, suggests specific perturbation in the SAM binding in these mutants.
Table 5: Steady state kinetic parameters for MoaA GG Motif Mutants

Steady State Kinetics for MoaA GG motif mutants with and without Wt Peptide. Complete summary of steady state kinetic parameters for GTP and SAM for MoaA GG Motif Variants with 0, 100, 500, or 800 µM 11-mer Wt peptide. $K_m$ and $k_{cat}$ determined by coupled assay with \textit{S. aureus} MoaC and HPLC analysis of cPMP. $K_d$ determined by anaerobic ITC. a- “No detection” - No cPMP detected by HPLC analysis. b- Not tested. c- Below the limit of detection for ITC analysis, 90 µM.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Peptide (μM)</th>
<th>Substrate</th>
<th>$K_i$ (μM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_s$ (μM)</th>
</tr>
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<tbody>
<tr>
<td>MoaA Wt</td>
<td>0</td>
<td>GTP</td>
<td>3.1 ± 0.67</td>
<td>0.042 ± 0.005</td>
<td>5.0 ± 3.0</td>
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<tr>
<td></td>
<td>0</td>
<td>SAM</td>
<td>5.1 ± 1.4</td>
<td>0.045 ± 0.007</td>
<td>1.7 ± 0.6</td>
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<tr>
<td>MoaA Δ330-340</td>
<td>0</td>
<td>GTP</td>
<td>N.D.*</td>
<td>N.D.*</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>GTP</td>
<td>88 ± 9.8</td>
<td>0.0067 ± 0.0003</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>GTP</td>
<td>36 ± 8.7</td>
<td>0.023 ± 0.002</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td>MoaA G339A</td>
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<td>GTP</td>
<td>N.D.*</td>
<td>N.D.*</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>GTP</td>
<td>30 ± 5.0</td>
<td>0.020 ± 0.0009</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>GTP</td>
<td>19 ± 1.8</td>
<td>0.045 ± 0.006</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td>MoaA G339S</td>
<td>0</td>
<td>GTP</td>
<td>N.D.*</td>
<td>N.D.*</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>GTP</td>
<td>70 ± 9.8</td>
<td>0.0087 ± 0.0004</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>GTP</td>
<td>19 ± 3.3</td>
<td>0.050 ± 0.003</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td>MoaA G340A</td>
<td>0</td>
<td>GTP</td>
<td>N.D.*</td>
<td>N.D.*</td>
<td>5.2 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>GTP</td>
<td>32 ± 6.9</td>
<td>0.0073 ± 0.00066</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>GTP</td>
<td>7.7 ± 3.8</td>
<td>0.047 ± 0.003</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td>MoaA G340S</td>
<td>0</td>
<td>GTP</td>
<td>N.D.*</td>
<td>N.D.*</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td></td>
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<td>GTP</td>
<td>210 ± 53</td>
<td>0.015 ± 0.002</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>GTP</td>
<td>52 ± 10</td>
<td>0.048 ± 0.004</td>
<td>&gt; 90°</td>
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<tr>
<td>D198A-MoaA</td>
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<td>GTP</td>
<td>6.9 ± 0.73</td>
<td>0.0046 ± 0.0015</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td></td>
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<td>SAM</td>
<td>36 ± 6.9</td>
<td>0.0039 ± 0.00074</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td>R330A, K331A,</td>
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<td>GTP, SAM</td>
<td>N.D.*</td>
<td>0.0043 ± 0.0004</td>
<td>&gt; 90°</td>
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<tr>
<td>K332A-MoaA</td>
<td>100</td>
<td>GTP, SAM</td>
<td>N.D.*</td>
<td>0.021 ± 0.007</td>
<td>&gt; 90°</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>GTP, SAM</td>
<td>N.D.*</td>
<td>0.035 ± 0.002</td>
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</table>
Figure 48: Anaerobic ITC Analysis for MoaA GG Motif Mutants

Representative plots of anaerobic ITC experiments using 35 μM MoaA in 20 mM Tris, pH 7.6, 150 mM NaCl, 5 mM DTT titrated with 350 μM substrates in an identical buffer. Summary of data listed below graphs. $K_m$ determined by coupled assay with S. aureus MoaC and HPLC analysis of cPMP. $K_d$ determined by anaerobic ITC. “No detection” - No cPMP detected by HPLC analysis. - Not tested. Below the limit of detection for ITC analysis, 90 μM. (a) 35 μM MoaA Wt titrated with 350 μM GTP. (b) 35 μM MoaA G339A titrated with 350 μM GTP. (c) 35 μM MoaA G340A titrated with 350 μM GTP. (d) 35 μM MoaA Wt titrated with 350 μM SAM. (e) 35 μM MoaA G339A titrated with 350 μM SAM. (f) 35 μM MoaA G340A titrated with 350 μM SAM. (g) 35 μM MoaA G339A with 11-mer. (h) 35 μM MoaA G340A with 11-mer.
and 0.5 mM 11-mer Wt peptide titrated with 350 µM SAM. (h) 35 µM MoaA G340A and 0.5 mM 11-mer Wt titrated with 350 µM SAM.

4.2.4. The Role of MoaA GG Motif in H-atom Transfer

The critical role of the GG motif in SAM binding suggested its potential involvement in the radical initiation process. In the MoaA catalysis, SAM is reductively cleaved to transiently generate 5’-dA•, which then abstracts H-atom from the 3’-position of GTP (Figure 43b) [61, 75]. Perturbation in the H-atom transfer step can be tested by monitoring kinetic isotope effects (KIE) using [3’-D]GTP or GTP as substrate. In my previous study, I reported a small KIE for wt MoaA reaction (1.28 ± 0.05) [61], suggesting the kinetic masking of the H-atom transfer. When G340A-MoaA was assayed in the presence the 11-mer wt peptide, a significantly greater KIE of 3.0 ± 0.30 was observed (Figure 49a). The > 2-fold increase in the KIE suggests that the H-atom abstraction is more rate limiting in in G340A-MoaA compared to the wt enzyme, suggesting the perturbation in the H-atom transfer step.

The perturbation in the H-atom transfer step was also suggested by the increased amounts of the abortive cleavage of SAM. Abortive cleavage is frequently observed in radical SAM enzymes, in which the formation of 5’-dA• by the reductive cleavage of SAM is uncoupled from the H-atom abstraction from substrate [193, 194]. In my in vitro assays of the wild-type enzyme, MoaA reaction is very well coupled and only minimal amount of uncoupling is detectable (4.1 ± 2.7 and 5.5 ± 3.0 % for GTP and [3’-D]GTP, respectively, Figure 49b). Comparable amount of uncoupling (8.2 ± 4.8%) was observed
for G340A-MoaA in the presence of the 11-mer Wt peptide when assayed using GTP with natural isotope abundance. However, when [3′-D]GTP was used, significantly greater value (25 ± 6.7 %) was observed. Together with the increased KIE in G340A-MoaA, these observations suggest that the perturbation in the GG motif significantly slows down the rate H-atom abstraction.

**Figure 49: GG Motif Coordinates SAM-binding and Facilitates Radical Initiation**

(a) KIE of wt MoaA vs G340A-MoaA (with 11-mer Wt peptide). KIE determined by comparing rate of cPMP formation with GTP vs. [3′-D]GTP using Wt-MoaA and G340A-MoaA. 3 µM MoaA enzyme assayed with 10 µM MoaC in 50 mM Tris, pH 7.6, 0.3 mM NaCl over 20 minutes at 25°C with 1 mM SAM, 1 mM DTH, 5 mM DTT, 2 mM MgCl₂, and 1 mM GTP or [3′-D]GTP. (b) The amount of abortive SAM cleavage observed in % relative to cPMP formation by wt MoaA and G340A-MoaA with 11-mer Wt peptide. GTP with natural isotope abundance (green bars) or with 3′-H labeled (grey bars) was used as substrate. In both panels, the assays were performed in the presence of MoaA (3 µM), MoaC (10 µM), NaCl (0.3 mM), SAM (1 mM), DTH (1 mM), DTT (5 mM), MgCl₂ (2 mM), GTP (1 mM) in 50 mM Tris, pH 7.6. 200 µM of the 11-mer wt peptide were used in the G340A-MoaA mutant assays. Reactions were triplicated, and error bars represent standard deviation.
4.2.5. Site of the C-terminal Tail Interaction

The characterizations described above suggested the critical role of the GG motif in SAM binding and the H-atom abstraction step. To obtain further insights into the mechanism by which the GG motif affects these processes, I sought for the site of interaction between the disordered C-terminus and MoaA. As described above, the activity restoration by the 5- and 8-mer peptides were 10-100-folds lower than that by the 11-mer peptide. The distinct activity restoration capabilities between 8- and 11-mer peptides suggested that the three cationic residues (RKK) at the N-terminus of the 11-mer peptide may be important for the peptide binding to MoaA. This hypothesis predicted the presence of electrostatic interaction between the three cationic residues of the peptide and surface exposed acidic residue(s) of MoaA. In the reported crystal structures [12, 71], four acidic residues were found within proximity of the last ordered residue at the C-terminus (Q329, Figure 43d). D198 and D206 are within proximity of the SAM binding site (Figure 43d, 44a), whereas E97 and D98 are removed. Therefore, I prepared MoaA double mutants harboring G340A and an additional Ala mutation in one of the acidic residues. The effects of the mutations in the acidic residues were evaluated by the peptide rescue assay. Intriguingly, D198A/G340A-MoaA showed significantly diminished activity even in the presence of saturating concentration of 11-mer peptide (0.8 mM). In this mutant, the activity restored by the 11-mer and 8-mer
peptides was essentially identical. On the other hand, the activities of the other double mutants were restored by 11-mer peptide to the extent comparable to that of wt-MoaA, and > 5-folds greater than those by the 8-mer peptide. These observations demonstrate that D198 and the three cationic residues in the peptide (RKK) are both critical for the activity restoration of the MoaA GG motif mutants.

Further evidence for the critical role of the interaction between D198 and R330/K331/K332 for MoaA catalysis and the GG motif function was obtained from characterization of D198A- and R330A/K331A/K332A-MoaA mutants. Both of the two mutants had diminished activity (8% that of wt MoaA, Table 5) comparable to that observed for G340A-MoaA complemented with the 8-mer peptide without the three cationic residues (RKK). Intriguingly, the activity of the R330A/K331A/K332A-MoaA triple mutant was restored by the 11-mer peptide. The peptide titration experiments showed a sigmoidal relation between the restored MoaA activity and the concentration of the peptide with $K_d = 0.17 \pm 0.030$ mM and Hill Coefficient $= 2.5 \pm 0.6$ (Figure 50), which are comparable to those observed in the peptide titration experiments for G340A-MoaA (Figure 47b, Table 5). The activity at the saturating concentration of the peptide was comparable to that of wt-MoaA ($k_{cat} = 0.035 \pm 0.0022$ min$^{-1}$). No significant activity restoration was observed by the 8- and 5-mer peptides (Figure 47c). The activity of D198A-MoaA was not restored by any of the peptides. These observations suggest that the presence of D198 and R330/K331/K332 in MoaA or in the C-terminal peptide is
essential for the activity of MoaA. In combination with the double mutant experiments, all the observations are consistent with the model where the C-terminal tail of MoaA interacts with D198 to orient the GG motif towards the SAM binding site.

Figure 50: Peptide Titration of RRK-MoaA GG Motif Mutant

Concentration-dependent restoration of activity of R330A, K331A, K332A-MoaA using 11-mer wt peptide. Rate of cPMP Formation, in min⁻¹, determined using 3 µM MoaA enzyme assayed with 10 µM MoaC in 50 mM Tris, pH 7.6, 0.3 mM NaCl over 20 minutes at 25°C with 1 mM SAM, 1 mM DTH, 5 mM DTT, 2 mM MgCl₂, 1 mM GTP, and specified concentrations of 11-mer wt peptide. Reaction tested in triplicated, and error bars represent standard deviation. Activity of G340A-MoaA in the presence of various concentrations of the 11-mer Wt peptide. The solid line is the non-linear fit to Hill equation [192] with $k_{cat} = 0.035$ min⁻¹, $K_D = 172$ µM, and Hill coefficient = 2.46.

4.3. Discussion

Here I have demonstrated a successful rescue of the catalytic activity for the GG mutants using synthetic peptides, and its use to investigate the function of the GG motif. My initial characterization of MoaA variants with mutations in the GG motif revealed no
significant structural perturbation, but complete depletion of the catalytic activity. When these mutants were incubated with synthetic peptides corresponding to the C-terminal tail of wt MoaA, almost complete restoration of the catalytic activity was observed with $K_d = 0.15$ mM. Further detailed characterizations suggested that the mutations in the GG-motif caused perturbations in SAM binding and the H-atom transfer from GTP to 5'-dA•. In addition, the investigation into the potential interaction site for the MoaA C-terminal tail suggested its interaction with D198 that locates adjacent to the SAM binding pocket. These observations in combination suggested that the GG motif constitutes a part of the active-site to provide affinity for SAM binding, and affects the rate of H-3’ atom abstraction from GTP by 5’-dA•.
Figure 51: Structural Comparison of Radical SAM Enzymes

Surface-representations of several Radical SAM enzymes. Illustrated are the SAM-binding sites. (a) Molybdenum Cofactor Biosynthesis Protein, MoaA [12]. (b) Pyruvate Formate-Lyase Activating Enzyme (PFL-AE) [195]. (c) [Fe-Fe] Hydrogenase Maturase Protein, HydE [196]. (d) Lysine Aminomutase (LAM) [197]. (e) Oxygen-independent Coproporphyrinogen III Oxidase, HemN [198]. (f) Biotin Synthase (BioB) [199].

My proposal for the C-terminal tail as a part of the active-site is also consistent with the previously reported crystal structures of MoaA [12, 71]. In these structures, where the C-terminal 11 amino acid residues are disordered, SAM is highly exposed to solvent (Figure 51a, 53a). This is a sharp contrast to the structures of other radical SAM enzymes (Figure 51) [70, 195-200]. Typically buried 5-10 Å from the nearest protein surface, the 4Fe-4S cluster and SAM molecule that generates the 5´-dA• is found deep in the active site of radical SAM enzymes and shielded from solvent that may
unproductively quench the radical during catalysis. Of the radical SAM enzymes with published SAM-bound structures, the only exceptions to this are MoaA and the oxygen-independent Coproporphyrinogen III oxidase, HemN (Figure 51b). It has been previously suggested that MoaA and HemN may undergo further conformational changes before catalysis to reduce the solvent accessibility in the environment surrounding the active site [70]. The structure of MoaA also revealed minimal interactions between the active-site residues and the adenine base of SAM (Figure 53b). This is once again in contrast to the highly coordinated binding of SAM seen in other radical SAM enzymes. In pyruvate formate-lyase activating enzymes (PFL-AE), for example, SAM is well-coordinated in the active site of enzyme by multiple hydrogen bonds and through pi stacking interactions with several adjacent residues (Figure 53d) and is buried within the enzyme (Figure 53c) [195]. It is important to note that the GG motif in MoaA was previously considered to be the site of glycyl radical formation. However, recent isotope tracing experiments by us and others [61, 75] were more consistent with direct abstraction of H-3’ of GTP by 5’-dA•. I also confirmed the deuterium transfer from 3’ of GTP to 5’-dA in the G340A-MoaA complemented with the 11-mer peptide, where nearly all of the detected 5’-dA had 2H incorporated (Figure 52). Based on these observations, I propose that the GG motif in MoaA constitutes a part of the active site and provides physical affinity to bind and stabilize SAM.
Figure 52: H-Abstraction Studies with MoaA GG Motif Mutants.

(a) Model Wt-MoaA mechanism with [3'-D]GTP if GG motif was acting as glycyl radical and directly abstracting the H-atom. With multiple turnovers of MoaA, 5'-dA would incorporate $^2$H and thus indistinguishable from my proposed mechanism of direct H-abstraction by the 5'-dA radical. (b) Model G340A-MoaA mechanism with [3'-D]GTP if GG motif was acting as glycyl radical and directly abstracting the H-atom. When tested with an excess of 11-mer Wt peptide, multiple turnovers of MoaA would likely not produce 5'-dA with $^2$H-incorporation as the peptide would freely disassociate from MoaA. This was not observed in my experiments, as isolated 5'-dA had $^2$H-incorporation. (c) Summary of LC-MS analysis from coupled reactions with 30 μM MoaA Wt or G340A with 60 μM MoaC Wt, substrates (0.5 mM SAM, and 0.5 mM GTP or 3'-D-GTP), and with or without 0.5 mM 11-mer peptide. Percent Labeling with $^{2}$H =
100 \times \frac{[\text{MS Intensity at } 253.115 \text{ m/z}]}{[\text{MS Intensity at } 253.115 \text{ m/z}]+[\text{MS Intensity at } 252.113 \text{ m/z}]}\). (d) Mass Spectrum of 5’-dA from LCMS experiment with MoaA Wt Reaction with 3’-D-GTP. (e) Mass Spectrum of 5´-dA from LCMS experiment with MoaA G340A Reaction with 3’-D-GTP, 11-mer peptide.

Considering the significant ambiguity remaining about the mechanism of initiation of radical reactions in radical SAM enzymes [201], my findings of the use of the C-terminal GG motif in MoaA in SAM binding with significant effects on H-atom transfer is intriguing. The role of protein conformational change in the control of radical initiation has been proposed for PFL-AE [68, 195], as well as other free-radical dependent enzymes. In the active form of MoaA, I propose that flexible C-terminus of MoaA allows for the GG motif to enter the solvent accessible SAM-binding pocket of MoaA (Figure 53e). This facilitates the binding of SAM and creates an environment favorable for radical initiation. After catalysis, the C-terminus likely exits the SAM-binding pocket, which enables the release of the MoaA products. In this way, the flexible C-terminal tail of MoaA acts as gating mechanism for the reaction. As was seen in the assays with my MoaA variants, mutations of the C-terminus to bulkier terminal residues and truncations prevent likely prevent entry into this sterically hindered binding pocket. Therefore, my findings with MoaA GG-motif add additional evidence for enzymes to use protein conformational dynamics to control the formation and the reactivity of free radical species. Because MoaA activity can be triggered by exogenous peptide supplementation, it may provide an opportunity to study this process and will likely provide important insights into the mechanism of radical SAM in general.
Figure 53: Catalytic Function of the GG Motif

(a) Solvent exposed SAM-binding pocket of *S. aureus* MoaA [12]. (b) Distances between the adenine of SAM and the active-site amino acid residues. (c) Buried SAM in the active site of the radical SAM enzyme, Pyruvate Formate-Lyase Activating Enzyme (PFL-AE)
(d) Bound-SAM is well-coordinated PFL-AE through hydrogen bonding several with adjacent active-site residues [195]. (e) Model for the function of the GG motif in MoaA catalysis. The flexible C-terminus allows for the GG motif to enter the solvent accessible SAM-binding pocket of MoaA, which in turn facilitates the binding of SAM and creates an environment favorable for radical initiation. Protected in the now enclosed active site, the reduced SAM-associated 4Fe-4S cluster generates a 5’-dA radical, and catalyzes the formation of 3’,8-cH2GTP. After catalysis, the C-terminus exits the SAM-binding pocket, which enables the release of the MoaA products.

4.4. Materials & Methods

Materials: Guanosine 5’-triphosphate (GTP), S-adenosyl-L-methionine (SAM), dithiothreitol (DTT), sodium dithionite were purchased from Sigma-Aldrich. Non-linear least square fitting of kinetic data was carried out using KaleidaGraph software (Synergy Software, Reading, PA). Anaerobic experiments were carried out in an UNIlab workstation glove box (MBaun, Stratham, NH) maintained at 10 ± 2 °C with an O2 concentration < 0.1 ppm. All HPLC experiments were performed on a Hitachi L-2130 Pump equipped with an L-2455 diode array detector, an L-2485 fluorescence detector, an L-2200 autosampler and an ODS Hypersil C18 column (Thermo Scientific) housed in an L-2300 column oven maintained at 40 °C. UV-vis absorption spectra were determined using the U-3900 UV-VIS ratio recording double-beam spectrometer (HITACHI).

Synthetic peptides were purchased from Genscript.

Expression and Purification of Wt-MoaA and MoaA Variants

*S. aureus* Wt-MoaA with a hexahistidine (His6) tag at the N-terminus was heterologously expressed, purified, and anaerobically reconstituted as previously described without modification [61]. MoaA variants with single point mutations were
prepared by following the Stratagene’s QuikChange site-directed mutagenesis protocol using the primers shown in Table 6 and pET-HisMoaA [61] as a template. The triple mutant, R330A/K331A/K332A-MoaA was prepared using pET-HisMoaA-R330A as a template.

Table 6: DNA Primers Used for Site-Directed Mutagenesis of MoaA.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence(5’to3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ330-340</td>
<td>GTGCCAATCGTCAATAACGTAAAGATAAAACATG</td>
</tr>
<tr>
<td>Δ336-340</td>
<td>CAACGTAAAGATAAAACATGTAATATTTGGTTGTAATG</td>
</tr>
<tr>
<td>D198A</td>
<td>GAATTATGGCTGCTTAATGATAATG</td>
</tr>
<tr>
<td>D206A</td>
<td>GAATTATGGCTGCTTAATGATAATG</td>
</tr>
<tr>
<td>D98A</td>
<td>GATGGATAGAGCAGATTGTTGGAC</td>
</tr>
<tr>
<td>E97A</td>
<td>CGATGATTTGCTGATATTGTTGGAC</td>
</tr>
<tr>
<td>G339A</td>
<td>CATGAAATATATTGGCTGCTTAATGATAATG</td>
</tr>
<tr>
<td>G339S</td>
<td>CATGAAATATATTGGCTGCTTAATGATAATG</td>
</tr>
<tr>
<td>G339V</td>
<td>CATGAAATATATTGGCTGCTTAATGATAATG</td>
</tr>
<tr>
<td>G340A</td>
<td>GAATTATGTTGTCTGAAATGATAATG</td>
</tr>
<tr>
<td>G340S</td>
<td>GAATTATGTTGTCTGAAATGATAATG</td>
</tr>
<tr>
<td>K331A</td>
<td>CAATCGTAAACGTGCTGCTTAATGATAATG</td>
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<tr>
<td>K332A</td>
<td>CAATCGTAAACGTGCTGCTTAATGATAATG</td>
</tr>
<tr>
<td>R330A</td>
<td>GCCAATCGTCAAGCGGACAGCGATAAAACATG</td>
</tr>
<tr>
<td>R330A--&gt;R330A,K331A,K332A</td>
<td>CAATCGTCAAACGAGCGGACAGCGATAAAACATG</td>
</tr>
</tbody>
</table>

In vitro characterization of MoaA

For the coupled assay, purified wt- or mutant MoaA (1.0 μM) was anaerobically incubated with MoaC (3 μM), GTP (1 mM), SAM (1 mM), and sodium dithionite (1 mM) in the assay buffer (50 mM Tris-HCl pH 7.6, 1 mM MgCl₂, 2 mM DTT and 0.3 M NaCl) at 25 °C for 60 min. The reaction was then quenched with 0.1 volume of 25% trichloroacetic acid (TCA), and cPMP was quantified by HPLC after its conversion to a fluorescent derivative, compound Z, as previously reported [61]. For activity rescue assays with
MoaA GG motif mutants, synthetic peptides in H₂O were included in the reaction mixture at specified concentrations. For the steady state kinetic analysis, assays were performed at 25 °C under anaerobic conditions by incubating Wt-MoaA (0.1 µM) or MoaA mutants (1.0 µM) with Wt-MoaC (5 µM), sodium dithionite (1 mM), and varied concentrations of SAM & GTP (0, 1, 2, 5, 10, or 50, 100 µM) in the assay buffer (50 mM Tris-HCl, pH 7.6, 0.3 M NaCl, 1 mM MgCl₂, 5 mM DTT). The reaction was initiated by the addition of MoaA. At each time point, an aliquot (90 µL) was removed and mixed with 10 µL 25% (w/v) TCA to quench the reaction. cPMP quantified as previously described [61].

**Anaerobic ITC**

Using a GE MicroCal VP-ITC under the flow of argon, anaerobic ITC was performed on wt-MoaA and MoaA GG motif mutants. 0.5 mL of 350 µM GTP, SAM, or pure degased buffer was titrated into 1.5 mL of 35 µM MoaA in 20 mM Tris, pH 7.6, 150 mM NaCl, 5 mM DTT over 29 injections, at 24°C, 307 rev per minute.

**Functional Characterization of MoaA with [3´-D]GTP**

Wt-MoaA and MoaA GG motif mutants were assayed for perturbations in H-abstraction using [3´-D]GTP synthesized as previously described [61]. Rate of cPMP formation was determined for Wt-MoaA and G340A-MoaA using GTP or [3´-D]GTP as substrate. In each assay, 3 µM wt or mutant MoaA was incubated with 10 µM MoaC, 0.3 mM NaCl, 1 mM SAM, 1 mM DTH, 5 mM DTT, 2 mM MgCl₂, and 1 mM GTP or [3´-
D]GTP in 50 mM Tris, pH 7.6 over 20 minutes at 25°C. KIE determined by comparing rate of reaction with GTP vs. [3’-D]GTP. Percent uncoupling, or abortive cleavage, of wt MoaA vs G340A-MoaA (with 11-mer Wt peptide) was determined using 20 μM MoaA enzyme assayed with 40 μM MoaC in 50 mM Tris, pH 7.6, 0.3 mM NaCl over 20 minutes at 25°C with 1 mM SAM, 1 mM DTH, 5 mM DTT, 2 mM MgCl₂, and 1 mM GTP or [3’-D]GTP. cPMP and 5’-dA were quantified by HPLC as previously described [61].

**Analysis of MoaA for PTM**

540 μg/mL MoaA in 50 mM ammonium bicarbonate, pH 8.0 was treated with 25 ug/mL Trpysin for 24 hours at 37°C while shaking. The digested sample was filtered with a 10,000 Da MWCO filter, lyophilized, and resuspended in pure ddH₂O for LC-MS analysis. 5 μL of 50 μM digested peptide was analyzed in positive ion-mode using an Agilent ESI-TOF-MS and Agilent Poroshell 120, C18, 2.1 x 75 mm, 2.7 μm column (Part No #697775-906). A linear gradient was employed at 0.3 mL/min using pure H₂O (Solvent A) and 100% ACN (Solvent B): 2-24% B for 20 min. Data was analyzed using Agilent Masshunter software.

**Anaerobic Size Exclusion Chromatography**

200 μL of 75 μM MoaA was analyzed at 4°C under anaerobic conditions by fast protein liquid chromatography (ATKA) using a GE Superdex 200 10/300 GL column (Part Number # 17-5175-01). An isocratic gradient at 3 mL/min was employed using an anaerobic buffer: 50 mM Tris, pH 7.6, 300 mM NaCl, 5 mM DTT, 1 mM MgCl₂. Protein
elution was monitored by absorbance at 280 nm. Elutions containing MoaA were collected under the flow of argon and analyzed by SDS-PAGE and for activity, as described above. For analysis in the presence of substrates, 0.1 mM GTP and SAM was added to the mobile phase. A similar protocol was used for analyzing the MoaA GG motif mutants.

Functional Characterization of MoaA with [3´-D]GTP

Wt MoaA and MoaA GG motif mutants were assayed for H-abstraction using [3´-D]GTP, which was synthesized as previously described[61]. Detection of ²H-incorporation was repeated as previously described [61], using LC-MS analysis on coupled reactions with 30 µM MoaA Wt or G340A with 60 µM MoaC Wt, substrates (0.5 mM SAM, and 0.5 mM GTP or 3´-D-GTP), and with or without 0.5 mM 11-mer peptide. Percent Labeling with 2H = 100 x ([MS Intensity at 253.115 m/z])/([MS Intensity at 253.115 m/z]+[MS Intensity at 252.113 m/z]).
5. Preliminary and Future Mechanistic Studies of MoaA and MoaC

For both MoaA and MoaC, I have proposed mechanisms for the formation of 3’,8-cH2GTP and cPMP, respectively. To provide support to both of these mechanistic hypotheses, the use of substrate analogs and enzyme mutations can be an invaluable biochemical approach to accumulate and structurally characterize biosynthetic intermediates. Similar efforts have been successful in probing the catalytic mechanisms of several seemingly complex enzyme systems [202-207]. In the case of MoaA and MoaC, by using substrate analogs and the aforementioned catalytic mutants, I have been able to employ a combination of techniques such as LCMS, chemical derivatization, and EPR to study accumulated nucleotide products.

5.1. Understanding the Catalytic Mechanism of MoaA

5.1.1. Using Substrate Analogs to Probe the Mechanism of MoaA

For MoaA, I have proposed that the reaction is initiated by the abstraction of H-3’ by 5’-dA•, generated by the reductive cleavage of SAM using the N-terminal 4Fe-4S cluster as a reductant (Figure 54a). The resulting C-3’ radical then attacks C-8 and generates an 3’,8-cycloGTP aminyl radical intermediate, which is then converted to 3’,8-cH2GTP. To test this mechanistic hypothesis, I designed and tested several GTP analogs predicted to inhibit specific steps of my hypothesized formation of 3’,8-cH2GTP (Figure 54b,c).
Figure 54: GTP Analogs for Probing the Mechanism of MoaA

(a) Proposed Mechanism of MoaA. (b) Guanosine 5’-triphosphate (GTP) and GTP substrate analogs that have and will be used with MoaA to assess the mechanism through product entrapment. (c) Relative formation of 3’,8-cH₂GTP analogs from corresponding GTP analogs by MoaA. 2’-dGTP, 2’-F-2’-dGTP, 7-Me-GTP, and GMPcPP all formed HPLC-detectible cyclic products when assays with MoaA, and were confirmed by anaerobic LC-MS. 3’-dGTP, 9-deaza-GTP, 8-Br-GTP, and XTP had no observed product formation, including 5’-dA, which suggests that MoaA may not accept this substrate analogs.
One useful group of mechanistic inhibitors are substrate analogs that traps the C-3′radical before cyclization to the 3′,8-cycloGTP aminyl radical intermediate (Step 2, Figure 4a). These pre-cyclization probes are informative in studying radical control and initiation in MoaA. In general, the mechanism of radical control in enzymes is a fundamental question that is largely unsolved, despite the prevalence of radical reactions in well-studied enzymes, such as P450 and ribonucleotide reductase [68, 204, 208]. As such, the use of GTP analogs with 2′ position systematically substituted with various halogens could provide clues to elucidate the lifetime of the 3′ radical formed by MoaA. Using this approach with analogs such as 2′-F-2′-deoxy-GTP and 2′-Cl-2′-deoxy-GTP, for example, you can incrementally change the bond dissociation energies of carbon-halogen bond (Figure 55). In the case of 2′-Cl-2′-deoxy-GTP with MoaA, the lower bond dissociation energy of the C-Cl bond facilitates the homolytic cleavage and formation of a dehydrated ribose product, as was recently demonstrated by Begley and colleagues (Figure 55b) [142]. This approach with *E. coli* RNR and 2′-Cl-2′-deoxyuridine 5′-diphosphate has also been successful [203].
General mechanism for MoaA with C2’-Substituted GTP Analogs: (a) 2’-Fluoro-GTP and (b) 2’-Chloro-GTP. Begley et al. recently determined that MoaA produces a dehydrated ribose product and liberated guanine through a homolytic cleavage of 2’-Chloro-GTP [142]. However, my work has demonstrated that 2’-fluoro-GTP generates no detectible shunt products, rather forms a halogenated cyclic product. This is likely because the C-F bond in 2’-Fluoro-GTP has a larger bond disassociation energy than the C-Cl bond in 2’-Chloro-GTP and thus favors the radical cyclization over homolytic cleavage.
In my preliminary anaerobic LC-MS analysis of standard MoaA reactions using the pre-cyclization probe, 2′-F-2′-deoxygen-GTP, I found that MoaA forms a cyclic product, 2′-F-2′-deoxygen-3′-8-ch₂GTP, without any major accumulation of a dehydrated ribose product (Figure 55a). As the C-F bond energy is actually 1.4 folds higher than C-O in the C2′-OH (488 vs 360 kJ/mol) [209], the fluoro-substituted analog as expected appears to be unable undergo homolytic cleavage in the MoaA reaction. This is in contrast to 2′-Cl-2′-deoxygen-GTP, as chlorine has a lower bond dissociation energy of 330 kJ/mol. While the reaction with 2′-F-2′-deoxygen-GTP only produced two folds less cyclic product than a typical reaction of MoaA with GTP after 120 minutes (Figure 54c), it was notably slower than the conversion of GTP to 3′-8-ch₂GTP (0.011 vs 0.045 min⁻¹, respectively). This reduced efficiency and slower rates of formation may be useful in the EPR detection of an accumulated radical species (Figure 56a), however further experiments are required.

Another useful group of mechanistic inhibitors are substrate analogs for MoaA that accumulate the 3′,8-cycloGTP aminyl radical intermediate prior to the formation of 3′-8-ch₂GTP (Step 3, Figure 4a). While I tested both 9-deaza-GTP and xanthine triphosphate, which have been reported to have slightly more negative redox potentials than GTP [210], neither generated any detectible reaction products. The redox potential of 7-deaza-GTP on the other hand is reported to be 0.3 V more negative than GTP [210], and thus may serve as a better probe for this of the reaction (Figure 56b). The absence of
N7 may also perturb the interaction between the R17 residues in MoaA and the substrate, thus slowing down the H+ transfer. Using this analog, a nucleotide base radical may be able to be trapped, and thus allowing for the final electron transfer step to be studied by EPR. These experiments could provide evidence for the role of the C-terminal 4Fe-4S. For example, if the radical is in equilibrium between the nucleotide base and 4Fe-4S cluster, then it maybe possible to determine the redox potential of the C-terminal cluster [205], which would provide some insight into its still unknown function.

Altogether though, future studies of these GTP analogs inhibitors for MoaA will provide strong evidence to support the proposed mechanistic hypothesis.

Figure 56: GTP Analogs for Studying the Radical Chemistry in MoaA
Proposed schemes for trapping and/or studying radical chemistry in Wt-MoaA (a) 2'-fluoro-GTP, despite forming cyclized product, could be useful in the EPR detection of an accumulated C3' radical species if the pre-cyclization step (step 2) is perturbed by the 2'-substitution. (b) 7-deaza-GTP may be a good chemical probe to trap and accumulate an aminal radical intermediate as it has a reported redox potential 0.3 V more negative than GTP [210]. (c) 7-methyl-GTP may also function as a pre- or post-cyclization probe of the reaction mechanism, in addition to be useful in studying the quenching of the aminyl radical in step 3 (see section 5.1.2.2).

5.1.2. Mechanistic Basis of MoCD in MoaA

Genetic mutations in human Moco biosynthetic enzymes are known to cause the fatal metabolic disorder, Moco deficiency, for which limited effective therapies are available [109, 110]. More than 50% of the identified genetic mutations found in Moco deficiency patients occur in the first step of the pathway --- the conversion of GTP into 3',8-cH₂GTP by the enzyme, MOCS1A [61, 75, 105, 187]. This unprecedented chemical transformation is catalyzed in bacteria by the highly conserved homolog of MOCS1A, MoaA [61]. While careful study of these genetic mutations in MoaA and MOCS1A may provide insights into the development more effective treatments for MoCD, they will undoubtedly act as a useful guide to understanding the mechanism of 3',8-cH₂GTP formation.

Of the mutations frequently found in MoaA that result in MoCD, a majority of these are located in the active site of MoaA and are thought to be critical for the catalytic activity [105]. For instance, mutations to any of the conserved cysteines that coordinate either of the two Fe-S clusters lead to a complete loss of activity [129]. The same is true of the three-conserved active site arginines surrounding GTP whose function are currently
unknown. Together, both sets of essential active-site residues in MoaA provide an excellent starting point to probe the catalytic mechanism of the enzyme.

![Figure 57: MoaA Active Site and MoCD Hot-spots](image)

Model of *S. aureus* MoaA active site created by overlaying the structures of MoaA in complex with GTP, and MoaA in complex with SAM [12]. Illustrated are the active-site residues frequently found mutated in MoCD patients (hot-spots). These include the active site arginines and cysteines ligating the two 4Fe-4S clusters, shown in yellow.

### 5.1.2.1. MoCD and the Critical Fe-S Clusters

MoaA is a radical SAM enzyme with an N-terminal SAM-binding 4Fe-4S cluster and an unusual C-terminal auxiliary 4Fe-4S cluster (Figure 57) [12]. While this C-terminal cluster has been shown to bind GTP, its redox function and role in catalysis is not well understood [71] [72]. In the previous crystal structures of MoaA, the C-terminal 4Fe-4S cluster aligns with the auxiliary clusters found in a recently discovered subset of
radical SAM enzymes, which are identified by their C-terminal SPASM/twitch motif [211, 212]. The redox functions of the auxiliary 4Fe-4S clusters in this subfamily have been studied mainly for radical SAM dehydrogenases that catalyze oxidation of alcohol and thiol into aldehydes in protein posttranslational modification [213] or antibiotic biosynthesis [144]. In these enzymes, the auxiliary 4Fe-4S clusters have been proposed to have electron transfer functions, based mainly on the structural orientation of the clusters [211, 212], but little enzymological evidence is available to support this hypothesis [214].

As mentioned above, mutations to any of the cysteines that ligate either of these two clusters in MoaA results in partial or complete loss of the activity of enzyme. In my current mechanistic model for MoaA, the 5’-dA • generates a C-3’ radical intermediate, which quickly undergoes an intramolecular radical cyclization by attacking C-8 of guanine to form an aminyl radical intermediate (Step 2, Figure 54a). This radical species is then reduced by the transfer of a proton and an electron to complete the reaction (Step 3). The source of electron in the final step to reduce the aminyl radical intermediate is currently unidentified. My present hypothesis is that the aminyl radical reduction is coupled to an oxidation of the C-terminal 4Fe-4S cluster from the 1+ oxidation state to 2+. This theory is supported by my estimations for the redox potential of 3’,8-cH:GTP (+0.17 ~ +0.22 V), which is based on the values reported for related molecules [215]. These values for 3’,8-cH:GTP are more positive than the redox
potentials of 4Fe-4S clusters structurally analogous to the C-terminal 4Fe-4S cluster of MoaA (-0.4 ~ -0.6 V) [160]. However, there is currently very limited experimental evidence for the redox properties of the C-terminal 4Fe-4S cluster during the catalytic turnover [72, 162]. I cannot eliminate the possibility that the C-terminal cluster functions only as a site of GTP binding with no redox function during catalysis. In such a case, the electron is supplied by an exogenous reductant, like dithionite or flavodoxin. Therefore, to distinguish these two possibilities, a major future aim would be to unambiguously demonstrate the redox properties of the two 4Fe-4S clusters.

To elucidate the role of the individual 4Fe-4S clusters, my current plan is to prepare MoaA with both clusters pre-reduced, and perform activity assays in the absence of exogenous reductant. The determination of the stoichiometry of the 4Fe-4S cluster oxidation and 3’,8-cH₂GTP formation under the single-turnover conditions will be fundamental to understanding the redox properties of the two 4Fe-4S clusters. Although the approach appears simple, it is expected that I may encounter several difficulties during these experiments, based on the previous studies of other SPASM/twitch radical SAM enzymes [155, 214, 216]. One of the major scientific obstacles appears to be the inability to detect the enzyme activity in the absence of exogenous reductant. This problem may be associated with the previous failures to prepare the enzymes with 4Fe-4S clusters in catalytically active redox states. Therefore, I designed experiments in a way that allows extensive troubleshooting.
In my preliminary characterization of MoaA, I surprisingly found that pre-reduced MoaA is catalytically active even in the absence of exogenous reductant. In these experiments, MoaA was first reduced using dithionite, which is then separated from MoaA by size-exclusion column chromatography. When this pre-reduced MoaA was assayed in the absence of reductant, it showed activity ~20% of that in the presence of excess reductant when assays in Tris-HCl buffer. In contrast, MoaA without the pre-reduction was not active in the absence of external reductant.

Figure 58 shows my preliminary EPR characterization of the pre-reduced MoaA in the absence of any substrate or reductant. In general, the \([4\text{Fe-4S}]^{1+}\) state is paramagnetic and detectable by EPR, whereas the \([4\text{Fe-4S}]^{2+}\) state is EPR silent. Therefore, the observed signals are likely associated with \([4\text{Fe-4S}]^{1+}\) clusters. The signal quantitation suggested the presence of 0.46 eq. of paramagnetic species per monomer of MoaA. The observed spectrum (black trace in Figure 58) was simulated (red trace in Figure 58) as a sum of two signals with axial symmetry (blue and green traces in 1 : 2 ratio) using the EasySpin software [217]. The g values for the major species (green trace, \(g_\parallel = 2.065, g_\perp = 1.894\)) were close to the g-values previously reported by the Hoffman and colleagues for the C-terminal 4Fe-4S cluster (\(g_\parallel = 2.063, g_\perp = 1.897\)) of a \(S. \text{aureus}\) MoaA mutant lacking the N-terminal cluster (MoaA-C24A/C28A/C31A) [72]. Although the g values for the N-terminal cluster are unknown, and therefore the assignment of the
minor species (blue trace, $g_\parallel = 2.034$, $g_\perp = 1.918$) requires further study, these preliminary results show the feasibility of my approach.

Figure 58: EPR Analysis of Pre-reduced MoaA

EPR spectrum of pre-reduced MoaA in the absence of substrates, products or exogenous reductant. Experimentally determined spectrum (black trace) was simulated (red) as a sum of two paramagnetic species (blue and green).

In future studies, I will continue to characterize the observed EPR signals by preparing MoaA mutants lacking one of the two clusters. This approach is likely achievable and has precedent for other 4Fe-4S cluster-containing proteins. As mentioned above, MoaA with only the C-terminal cluster has been reported [72], and my previous characterization of apo MoaA (< 0.1 eq. of 4Fe-4S clusters), MoaA was stable in the absence of 4Fe-4S clusters at 4 °C for more than a few hours [61]. The prepared MoaA
variants will be first characterized for the catalytic function by the standardized MoaA assay [61]. As they are expected to be catalytically inactive, the affinity of SAM and GTP binding will also be assessed using anaerobic isothermal calorimetry (ITC). MoaA with only the C-terminal cluster will be capable of binding GTP comparable to the wt enzyme, but will have diminished affinity to SAM. MoaA with only the N-terminal cluster will have the opposite characteristic. EPR characterization of the MoaA variants will be performed using X-band (9.4 GHz) EPR spectrum at 10 K. In addition, EPR spectra of MoaA wt and its mutants can be obtained the presence of substrates (GTP or SAM) or products (5′-dA, methionine, or 3′,8-cH2GTP). In general, 4Fe-4S clusters of radical SAM enzymes change their EPR spectra upon substrate binding [69, 218]. Such shift in EPR spectra could complicate the analysis of EPR spectra in the multiple turnover conditions. Therefore, it is important to understand the potential effects of ligands of 4Fe-4S clusters on the EPR spectra.

After the experiments described above, the EPR spectra of MoaA under single turnover conditions will be determined. In this experiment, pre-reduced MoaA will be incubated with substrates in the absence of any reductant, and the reaction will be quenched by freezing in liquid nitrogen. Hand freeze quenching will be sufficient considering the slow rate of catalysis (0.05 min⁻¹). EPR spectra will be determined for each sample, and the total concentrations of [4Fe-4S]⁺ clusters will be determined based on the total signal intensity. The ratio between the N- and C-terminal [4Fe-4S]⁺ clusters
will be determined based on the EPR spectral simulation as described above. These data can then be compared with the amount of product, 3’-8-CH₂GTP, formed at each time point, ultimately allowing us to correlate the progress of the reaction with the change in the amount of N- and C-terminal [4Fe-4S]¹⁺ clusters.

In a parallel effort, pre-reduced MoaA can be assayed in the presence of redox mediators with various redox potentials (Figure 8). If the reduction of the C-terminal cluster (estimated $E^o = -0.4 \sim -0.6$ V) is required, the activity of MoaA will require redox mediators with potentials more negative than -0.4 V. If the aminyl radical (estimated $E^o = +0.17 \sim +0.22$ V) is reduced directly by the exogenous reductant, MoaA activity will be observed even with the redox mediators with redox potentials more positive than -0.4 V. Such experiments have been successful in probing the redox function in isoprenoid biosynthetic enzymes [219]. Altogether, these studies will provide comprehensive view of the redox reaction catalyzed by MoaA, and detailed insights into the functions of the two 4Fe-4S clusters of MoaA.

5.1.2.2. MoCD and the Active-site Arginines

Another group of conserved residues found mutated in MoCD patients are three active site arginines of MOCS1A [129]. In S. aureus MoaA, these residues are R17, R266, R268, and are all adjacent to and with hydrogen bonding distance from the guanine of GTP (Figure 57) [12]. Interestingly, these arginine residues have been shown to affect only the catalytic activity of MoaA, but not the overall affinity of the enzyme towards
GTP [71]. As such, Schindelin and colleagues proposed that this triple arginine motif may play a key role in catalysis or in the positioning for GTP for the radical chemistry. However, enzymology support of this hypothesis was absent. Thus, the actual role of these residues and their contribution to 3’,8-cH₂GTP formation is currently unknown.

To elucidate the function of these arginine residues, I generated single point mutations of R17 to Ala, Lys, Leu, Gln, or Tyr in *S. aureus* MoaA. All of these R17 MoaA mutants were heterologously expressed in *E. coli* similar to the wild-type enzyme, and subsequently purified and anaerobically reconstituted to give holo-proteins with > 95% purity and an average 1.9 ± 0.12 4Fe-4S clusters per monomer. In my assessment of the each of the activities of these enzymes, only R17K-MoaA had any detectible activity ($k_{cat}$ = 0.055 min⁻¹). This mutant exhibited a 6.6 fold increase in the $K_m$ value for GTP compared to the Wt enzyme, similar to the catalytically-inactive R17A-MoaA mutant that was previously reported to have a ~ 2-3 fold increase the $K_a$ value for GTP [71]. These observations seem to suggest that a charged residue like lysine or arginine at residue 17 is required for MoaA activity, and may play a more significant role than just coordinating GTP binding.

Based on my current mechanistic hypothesis for MoaA, R17 could be electrostatically stabilizing or supplying a proton to the 3’,8-cycloGTP aminyl radical in order to form 3’,8-cH₂GTP. To test this hypothesis, a future experiment could include testing R17A-MoaA with the GTP analog, 7-methyl-GTP (Figure 56c). Based on my
mechanism, this mutant could form a 7-methyl-3’,8-cycloGTP cationic aminyl radical, which would then be further reduced without a proton transfer to 7-methyl-3’,8-cH₂GTP. If R17 is involved directly in the proton transfer to the aminyl radical, then R17A-MoaA should still be able to form 7-methyl-3’,8-cH₂GTP from 7-methyl-GTP.

Additional future experiments would likely include the X-band EPR analysis of the MoaA reaction with R17, R266, R268 mutations to the enzyme. Since the active-site mutations in enzymes that catalyze radical reactions often result in accumulation of radical intermediates [205, 206], this could be a useful way to probe the roles of each of these arginines in catalytic mechanism of MoaA. All together, these preliminary studies of the mutations commonly found in MoCD patients has provided valuable additional insights into the mechanism of MoaA, and allow for several promising future studies.

5.2. Understanding the Catalytic Mechanism of MoaC

5.2.1. Using Substrate Analogs to Probe the Mechanism of MoaC

Based on my current mechanistic hypothesis of MoaC, 3’-8-cH₂GTP undergoes a hydrolysis of the aminal moiety followed by the ring expansion rearrangement (Figure 59a). The last step is proposed as a coupled cleavage and cyclization of the triphosphate moiety. To support this hypothesis, each of these steps will be individually assessed using either competitive or mechanism-base inhibitors to MoaC (Figure 59b). These analogs will allow me to further probe the reactivity of MoaC by looking for entrapped products through anaerobic LC-MS, chemical derivatization, and crystallography with
Wt-MoaC. Eventually, the reactions with analogs can be scaled-up, and the entrapped products can be isolated and characterized by NMR spectroscopy for more definite characterization of the intermediate.

As illustrated above, I have had preliminary success in using MoaA to catalyze the conversion of several GTP analogs into corresponding analogs of 3’,8-cH\(^2\)GTP (Figure 54c). Using this approach, future mechanistic studies with MoaC will include the use of various 2’-, 3’-, or 7-substituted and non-hydrolyzable 3’,8-cH\(^2\)GTP analogs: 2’-deoxy-3’,8-cH\(^2\)GTP, 2’-F-2’-deoxy-3’,8-cH\(^2\)GTP, 3’-F-3’,8-cH\(^2\)GTP, 7-deaza-3’,8-cH\(^2\)GTP, 7-methyl-3’,8-cH\(^2\)GTP, and 3’,8-cH\(^2\)GMPcPP. More specifically, 7-deaza-3’,8-cH\(^2\)GTP is useful probe for the timing of the hydrolysis of the aminal moiety 3’,8-cH\(^2\)GTP. As the analog is likely to not undergo this hydrolysis in the first step of the proposed mechanism, the competitive inhibitor could be assayed with MoaC and analyzed further by anaerobic LC-MS and chemical derivatization to support the timing of this first step.

In contrast to the 7-substituted cyclic substrates, aminyl hydrolysis will likely still occur in 2’- and 3’-substituted 3’,8-cH\(^2\)GTP analogs, but not the deprotonation of the 2’-OH and 3’-OH groups required for the subsequent ring expansion. As a result, the 2’- and 3’-substituted 3’,8-cH\(^2\)GTP analogs may entrap a hydrolyzed aldehyde-containing intermediate with an expected 18 Da larger molecular weight compared to the starting molecule, and thus can be detected by anaerobic LC-MS. If the trapped molecules are unstable under the conditions of analysis, I will derivatize them using O-(p-nitrobenzyl)-
hydroxylamine (NBHA). NBHA derivatizes aldehydes and ketone molecules into oxime, allowing their stabilization and detection by HPLC and LC-MS [220].

The timing of the cyclic phosphate formation will be assessed by the use of a non-cleavable 3’,8-cH₂GTP analog: 3’,8-cyclo-7,8-dihydro-guanosine-5’-[(α,β)-methylene]triphosphate (3’,8-cH₂GMPcPP, Figure 59b). 3’,8-cH₂GMPcPP is expected to be resistant to the cyclic phosphate formation, and allow capture of an intermediate prior to the cyclic phosphate formation (Figure 59c). In my preliminary analysis, as expected I observed that 3’,8-cH₂GMPcPP acts an inhibitor of MoaC with an IC₅₀ value of 0.14 µM when assayed with 0.1 µM Wt-MoaC and 15 µM 3’,8-cH₂GTP. While the current mode of inhibition is unknown, this IC₅₀ value is significant compared to the Kₘ value for 3’,8-cH₂GTP of 0.21 µM. Furthermore, the Wt-MoaC and 3’,8-cH₂GMPcPP reaction yields an entrapped MoaC intermediate that can be chemically derivatized and shown to have a pterin moiety. This observation indicates that the cyclic phosphate formation may indeed be one of the last steps of cPMP formation as has been proposed in my mechanistic hypothesis.
Figure 59: 3′,8-cH₂GTP Analogs for Probing the Mechanism of MoaC

(a) Proposed Mechanism of MoaC. (b) 3′,8-cH₂GTP and 3′,8-cH₂GTP substrate analogs that have and will be used with MoaC to assess the mechanism through product entrapment. (c) Expected MoaC reaction with the non-hydrolyzable 3′,8-cH₂GTP substrate analog, 3′,8-cH₂GMPcPP.

5.2.2. Using MoaC Active-Site Mutants to Probe the Mechanism of MoaC

For the detailed mechanism for MoaC in Chapter 3, each of the identified active site residues are suggested to have an independent role in the conversion of 3′,8-cH₂GTP to cPMP. These conserved residues are critical to the activity of the enzyme and include the following: K51, H77, E112, E114, D128, K131. Using these residues to make catalytically impaired mutants of MoaC, I have recently begun to study entrapped
nucleotide intermediates of the MoaC reaction using anaerobic LC-MS, chemical
derivatization, and in situ NMR.

To probe my proposed mechanism of MoaC using these catalytically impaired
mutants, I developed an anaerobic HPLC method to directly analyze underivatized
intermediates and products of the MoaA and MoaC reactions (Figure 60). Using this
approach, 3′,8-cH₂GTP was first be produced in a MoaA reaction (Figure 60, Trace 1)
and then subsequently incubated with Wt-MoaC or a MoaC variant, such as K131A.
From the reaction with Wt-MoaC, all of the detectible 3′,8-cH₂GTP was converted into
cPMP after a 60 minute incubation (Figure 60, Trace 2). In the K131A-MoaC reaction, a
novel oxygen-sensitive intermediate accumulated and cPMP and 3′,8-cH₂GTP were no
longer detected (Figure 60, Trace 3). This new product was separated from K131A-MoaC
by filtration, and treated with Wt-MoaC to form cPMP (Figure 60, Trace 4), which
suggested that this molecule may indeed be an on-pathway intermediate of MoaC.
When K131A-MoaC is assayed with 3′,8-cH₂GMPcPP as a substrate analog, an oxygen-
sensitive intermediate accumulates with an identical UV-Vis absorption spectrum. This
supports the idea the base modification occurs before the cyclization of the phosphate
group.

In additional studies of the accumulated K131A-MoaC intermediate, LC-MS
analysis indicated that the molecule had an 18 Da larger molecular weight compared to
3′,8-cH₂GTP, which suggested the addition of water as was proposed in my mechanistic
hypothesis (Figure 60c). Characterization by chemical derivatization demonstrated that
the intermediate is still capable of forming DMPT, which further implies the presence of
an acid-labile 6-hydroxy-2,4,5-triaminopyrimidine moiety like 3',8-cH2GTP. All together,
these preliminary observations are consistent with the current model for the MoaC
catalysis through the formation of an aldehyde intermediate, and then a cyclization of
the phosphate group. Future studies will use additional combinations of catalytically
impaired mutants and substrates analogs to trap any other stable intermediates, and
continue probe the mechanism for cPMP formation by MoaC.
Figure 60: Preliminary Results from Product Entrapment with K131A-MoaC

(a) General anaerobic assay setup and (b) HPLC analysis for product entrapment experiments. Wt-MoaA was incubated with GTP and SAM for 60 minutes to produce 3’,8-cH2GTP (HPLC trace 1). This reaction sample was then be incubated with Wt- or K131A-MoaC to produce cPMP (HPLC trace 2) or an unknown oxygen-sensitive MoaC intermediate (HPLC trace 3), respectively. The K131A-MoaC incubated sample was subsequently filtered to remove these enzymes, and incubated with Wt-MoaC, which produced cPMP and consumed the unknown intermediate. Chromatography monitored at 322 nm. (c) Expected K131A-MoaC reaction with 3’,8-cH2GTP based on product entrapment, chemical derivatization, and LC-MS analysis.

5.3. Materials & Methods

Substrate Analogs for MoaA
Adenosine 5’-trisphosphate (ATP), Xanthosine 5’-trisphosphate (XTP), 7-methylguanosine-5’-trisphosphate (7-methyl-GTP), and 2’-deoxy-guanosine-5’-trisphosphate (2’-deoxy-GTP) were purchased from Sigma-Aldrich. Guanosine-5’-[(α,β)-methyleneo]triphosphate (GMPcPP) was obtained from Jena Biosciences. The nucleosides 3’-deoxy-guanosine and 9-deaza-guanosine were purchased from Sigma-Aldrich and Barry and Associates, respectively. 2’-fluoro-2’-deoxy-guanosine and 8-Br-guanosine were purchased from Alfa Aesar. These commercially available nucleosides were chemically phosphorylated [175] to yield the remaining GTP analogs.

**HPLC Analysis of MoaA Reactions with Substrate Analogs**

Enzyme activity assays were performed at 25 °C under anaerobic conditions by incubating MoaA (40 μM) with SAM (1 mM), sodium dithionite (1 mM), and the GTP analog (1 mM) in the assay buffer (50 mM Tris-HCl, pH 7.6, 0.3 M NaCl, 1 mM MgCl₂, 5 mM DTT). The reaction was initiated by the addition of MoaA. At each time point, an aliquot (90 μL) was removed and mixed with 10 μL 25% (w/v) TCA to quench the reaction. DMPT quantified as described previously [61].

**Anaerobic HPLC Analysis of Underivatized MoaA and MoaC Reactions**

Enzyme activity assays were performed at 25 °C under anaerobic conditions by incubating MoaA (40 μM) with SAM (1 mM), GTP (1 mM), and sodium dithionite (1 mM) in the assay buffer. The reaction was initiated by the addition of MoaA. After 60 minutes, Wt or K131A-MoaC (120 μM) was added and the reaction was incubated for
another 120 minutes, and quenched with 1:1 dilution with an anaerobic solution of 100% MeOH to remove protein. 60 µL of supernatant was analyzed by anaerobic HPLC using an Alltech Apollo C18 4.6 x 250 mm, 2.7 µm column (Part No #36511). A stepwise linear gradient was employed at 1.0 mL/min using anaerobic 0.1 M KH₂PO₄, pH 6.0, 8 mM tetrabutylammonium hydrogen sulfate (Solvent A) and 0.1 M KH₂PO₄, pH 6.0, 8 mM tetrabutylammonium hydrogen sulfate, 30% (v/v) ACN (Solvent B): 0% B for 6.5 min, 0-20% B for 6.5 min, 20-40% B for 13 min, 40-100% B for 8 min. Chromatography was monitored by absorbance at 253 and 320 nm.

**Anaerobic LC-MS Analysis of MoaA and MoaC Reactions**

For LC-MS analysis, enzyme activity assays were setup as described above, but in 50 mM ammonium bicarbonate, pH 8.0, as an assay buffer. Reactions were quenched after 120 minutes by filtered with a 10,000 Da MWCO filter. 10 µL of filtered reaction samples were analyzed anaerobically in positive ion-mode using an Agilent ESI-TOF-MS and Agilent Poroshell 120, C18, 2.1 x 75 mm, 2.7 µm column (Part No #697775-906). A stepwise linear gradient was employed at 0.15 mL/min using anaerobic 30 mM ammonium formate, pH 4.5 (Solvent A) and 100% MeOH (Solvent B): 0-2% B for 10 min, 2-50% B for 7 min, 50-100% B for 5 min. Data was analyzed using Agilent Masshunter software.

**EPR Analysis of Pre-Reduced MoaA**
2 mL of 750 μM of MoaA in 50 mM Tris-HCl, pH 7.6, 0.3 mM NaCl, and 2 mM DTT was incubated with 1 mM sodium dithionite for 25 minutes at 25 °C under anaerobic conditions to pre-reduce the 4Fe-4S clusters. Excess reductant was removed by anaerobic gel filtration using a Sephadex G-25 column (1.5 x 60 cm, 40 mL) equilibrated with in 50 mM Tris-HCl, pH 7.6, 0.3 M NaCl, 10% (v/v) glycerol. To test the efficiency of the pre-reduction, MoaA was diluted to 6 μM, and incubated with GTP (1 mM) and SAM (1 mM), and analyzed for DMPT formation. As a positive control, 1 mM sodium dithionite could be added to this pre-reduced MoaA. EPR analysis of pre-reduced 210 μM MoaA was recorded at 7 K and 2 mW power with a modulation amplitude of 10 G on a Bruker ESP 300 spectrometer equipped with an ER 041 MR microwave bridge and an ST4102 X-band resonator (Bruker). Sample temperature was maintained with an ITC503S temperature controller and an ESR900 liquid helium cryostat (Oxford Instruments).

Cloning, Expression, and Purification of Staphylococcus aureus R17A-MoaA Variants

The S. aureus R17A-MoaA variant was prepared by following the Stratagene's QuikChange site-directed mutagenesis protocol using pET-HisMoaA as a template and SDM primers [R17A-f (GACGTCCCATCCGTGACTTAGCGTTATCTGTGACAGATC), R17A-r (GATCTGTCACAGATAACGCTAAGTCACGGATGGGACGTC)]. R17A-MoaA was heterologously expressed, purified, and anaerobically reconstituted as previously described without modification [61].
HPLC Analysis of R17A-MoaA Reactions

R17A-MoaA activity assays were performed as a Wt-MoaC coupled assays at 25 °C under anaerobic conditions by incubating R17A- or Wt-MoaA (0.5 μM) and MoaC (5 μM) with SAM (1 mM), sodium dithionite (1 mM), and GTP (0, 0.5, 1, 2, 10, or 50 μM) in the assay buffer. The reaction was initiated by the addition of MoaA. At each time point, an aliquot (90 μL) was removed and mixed with 10 μL 25% (w/v) TCA to quench the reaction. cPMP and DMPT quantified as described previously [61].
6. Summary of Work

6.1. Introduction to the Problem

The molybdenum cofactor (Moco) is a redox cofactor found in almost all organisms and plays a central role in many medicinally important processes [5, 9]. For example, in pathogenic bacteria, Moco is critical to the virulence [112, 118, 119]. A recently reported antituberculosis agent was shown to target one of the Moco biosynthetic enzymes [103]. In humans, the cofactor is essential for the healthy development of the central nervous system [9]. Defects in human Moco biosynthesis leads to Moco deficiency (MoCD), a disease marked by neurological abnormalities and early childhood death. As such, further understanding Moco biosynthesis is important for the future development of novel therapies to treat MoCD or bacterial infections.

In all organisms, Moco must be synthesized de novo in cells as it is chemically unstable, particularly under aerobic conditions, and cannot be taken up as a nutrient. During Moco biosynthesis, the characteristic pyranopterin ring is constructed by a complex rearrangement of GTP into cPMP (Figure 61a) [11]. This unusual transformation involves the insertion of the guanine C-8 between C-2’ and C-3’ of ribose [10]. While two enzymes, MoaA and MoaC, have been known to be responsible for this step [10, 12, 123], their individual functions and catalytic mechanisms have been under significant debate. MoaA belongs to the radical SAM (S-adenosyl-L-methionine) superfamily. Enzymes in this superfamily catalyze the reductive cleavage of SAM using
oxygen sensitive Fe-S clusters, and transiently generate a 5’-deoxyadenosyl radical (5’-dA•), which then abstracts a H-atom from the substrate to initiate radical reactions. MoaC, on the other hand, does not show significant amino acid sequence homology to other functionally characterized enzymes. Given that significant ambiguity remained, my thesis project focused on providing insights into the mechanisms of the MoaA and MoaC-catalyzed conversion of GTP to cPMP.

6.2. Functional Characterization of MoaA & MoaC

Because of the complex chemistry required to form cPMP from GTP, previous studies have largely focused on MoaA based on the hypothesis that it catalyzes the majority of the rearrangement reaction by a mechanism involving free radical chemistry [12, 74, 75]. In line with such a view, MoaA was proposed to catalyze the conversion of GTP into pyranopterin triphosphate [75, 176]. Still, the experimental demonstration of this model has been challenging due to the poor stability of the MoaA product especially in the presence of oxygen. Additionally, previous in vitro studies failed to demonstrate both the order of the reaction and whether GTP could be converted into cPMP in a stepwise manner. To address this remaining ambiguity, I tested the individual catalytic activities of MoaA and MoaC, and found that MoaA alone is capable of converting GTP into an oxygen sensitive molecule. After extensive efforts, I successfully isolated the MoaA product from experiments performed under strict anaerobic conditions [61]. NMR characterization suggested its structure as 3’,8-cH₂GTP (Figure 61a). 3’,8-cH₂GTP
was converted to cPMP by MoaC or its human homologue, MOCS1B, with high specificities, suggesting the physiological relevance of 3',8-chGTP. This observation was in sharp contrast to the previous hypothesis, as it suggested that the pyranopterin ring is constructed by MoaC, and not MoaA.

**Figure 61: In Summary - in vivo and in vitro Functional Characterization of MoaC**

Overall summary of *in vivo* and *in vitro* functional characterization of MoaC. (a) Moco biosynthesis. (b) *In vitro* activity of MoaC wt and mutants as determined by a coupled assay with MoaA. (c) Steady state kinetic parameters determined for the conversion of 3',8-chGTP into cPMP. * Only the upper limit (1 µM) was determined because the reaction rate became impractically low below this substrate concentration. (d) Moco production in *E. coli* ∆moaC expressing MoaC wt or mutants.

### 6.3. In vitro and In vivo Studies of MoaC

While the characterization of 3',8-chGTP suggested the unprecedented catalytic function of MoaC to catalyze complex rearrangement reaction, the catalytic mechanism of MoaC was largely unknown due to the absence of amino acid sequence homology of MoaC to other functionally characterized enzymes, and the absence of crystal structures
in complex with its physiological ligands [76-79]. Therefore, to facilitate my mechanistic characterization, I solved the crystal structures of MoaC in complex with its physiological substrate (3′,8-CH2GTP) and product (cPMP). To capture structures of MoaC in complex with the substrate, 3′,8-CH2GTP, its catalytic activity has to be suppressed to prevent substrate consumption during crystallization. Since MoaC uses no co-substrate or cofactors that regulate its activity, I sought to identify MoaC mutants with diminished catalytic activities that still possess near to normal substrate affinity. Looking at strictly conserved residues in MoaC homologs from all kingdoms of life, I chose ten conserved residues, and individually mutated to Ala. The activities of these mutants were first assessed in vitro using purified enzymes. These assays revealed six mutants with activities less than 1% of wt MoaC (Figure 61b). Notably, these six amino acid residues were previously found clustered in the conserved pocket of the apo-form of MoaC [78]. Of the six mutants, three (K51A, D128A, and K131A) exhibited < 0.2% $k_{cat}$ relative to that of wt-MoaC with no detectable change in $K_m$, suggesting that substrate binding in these mutants is not significantly perturbed (Figure 61c).

To demonstrate the physiological relevance of the in vitro characterizations, I performed functional complementation of a Moco-deficient E. coli strain lacking the wt moaC gene (E. coli ΔmoaC). Due to the essential role of Moco in nitrate reductase (NR) catalysis, the E. coli ΔmoaC strain does not express functional NR and exhibits significant growth defects under anaerobic nitrate respiration conditions [78, 177]. Genetic
complementation of this strain with wt-MoaC rescued Moco biosynthesis as assessed by NR activity and bacterial growth under nitrate respiration conditions. On the other hand, complementation with mutant moaC genes exhibited poor growth under nitrate respiration conditions, and very low or no detectable NR activity (Figure 61d). These observations indicate that MoaC residues K51, D128, and K131 are important for the in vivo synthesis of Moco, supporting the physiological relevance of the in vitro activity assays.

In collaboration with Dr. Maria Schumacher at Duke University, I obtained structures of MoaC in complex with 3’,8-cH₂GTP using the catalytically deficient K51A-MoaC mutant (Figure 62a-b, 1.78 Å resolution, R_work/R_free = 17.38%/19.35%, PDB ID 4PYA). In the K51A-MoaC•3’,8-cH₂GTP structure, the ligand forms extensive H-bonding interaction with catalytically essential and strictly conserved amino acid residues providing high specificity in 3’,8-cH₂GTP binding by MoaC. Together with the in vitro and in vivo characterization of wt and active-site mutants of MoaC, all evidence supports 3’,8-cH₂GTP as the most likely physiological substrate of MoaC. To obtain further insight into this mechanism, I solved the structure of wt MoaC in complex with cPMP (Figure 62c, PDB ID 4PYD). In this structure, density corresponding to cPMP was located in the same pocket as 3’,8-cH₂GTP, providing further support that this pocket corresponds to the MoaC active site. In conclusion, my in vivo and in vitro functional characterization, and X-ray crystallography characterization of MoaC provide strong
evidence that 3’,8-cH₂GTP is most likely the physiological substrate of MoaC. Considering the strict conservation of the MoaC catalytic residues identified in this study, the function and mechanism of MoaC homologs is likely conserved among all kingdoms of life, including humans and bacteria.

Figure 62: In Summary – Structural Studies of MoaC in Complex with 3’,8-cH₂GTP or cPMP.

Crystal structures of MoaC in complex with 3’,8-cH₂GTP or cPMP. (a) The hexameric structure (a trimer of homodimers) of the K51A-MoaC in complex with 3’,8-cH₂GTP. 3’,8-cH₂GTP (shown in sticks) was found at the N-terminus of helix α4 (highlighted in black). b and c, The active-sites of K51A-MoaC in complex with 3’,8-cH₂GTP (b), and wt-MoaC with cPMP (c). Simulated annealing omit maps for the ligands (mesh) were calculated with F₀ – Fc coefficients contoured at 3 σ. H-bond interactions are shown as indicated by dashed lines with the distances indicated.
6.4. Functional Characterization of the C-terminal Tail of MoaA

In my studies of MoaA, I became interested in the fact that all MoaA homologs in prokaryotes and eukaryotes have two sequential glycine residues (G339 and G340) at the end of C-terminus (GG motif). The GG motif was first identified based on the study of Moco deficiency, where mutations in the GG motif of MOCS1A, the human homolog of MoaA, causes the disease [9]. The significance of the GG motif for function in humans was shown by heterologous expression of MOCS1A in E. coli lacking moaA gene [83]. While wt MOCS1A complemented the Moco biosynthesis in E. coli ΔmoaA strain, MOCS1A with mutations in the GG motif did not. Although this study demonstrated the importance of the GG motif, the function of the motif remained elusive due to a lack of structural and enzymological characterization.

As the first step of characterization, I prepared MoaA variants with mutations in the GG motif at either the conserved G339 or G340. Variants tested included single point mutations of Gly to Ala, Ser, Val, or 5 or 11 amino acid truncations at the C-terminus producing Δ336-340 and Δ330-340 MoaA, respectively. All mutants were heterologously expressed in E. coli, purified and anaerobically reconstituted to give holo-proteins with > 90% purity and 1.7 ± 0.3 4Fe-4S clusters per monomer. However, none of the mutants were catalytically active judged based on the formation of 3´,8-cH₂GTP (Figure 63b). In these reactions, no SAM cleavage was observed either. Since wt-MoaA catalyzes slow cleavage of SAM even in the absence of GTP, the lack of SAM cleavage in the GG motif
mutants suggested the perturbation in the radical initiation process. This hypothesis was further tested by in vitro studies of these mutants using a deuterium-labeled GTP substrate. The results of these experiments suggested that the H-abstraction and thus radical initiation step of MoaA was significantly perturbed in all of the tested GG mutants.

To provide more evidence for the function of this elusive GG motif, I developed an in vitro assay to directly rescue the GG mutant activity. In the crystal structures of MoaA, the C-terminal 11 amino acids (RKKINMNYIGG), including the GG motif, are disordered (Figure 63a). Therefore, I first hypothesized that the C-terminal region of MoaA is conformationally flexible, and its interaction with the remaining part of MoaA is essential for the activity of MoaA. To test this hypothesis, I prepared synthetic peptides that corresponded to the C-terminus of MoaA and tested their ability to restore the catalytic activity of the GG motif mutants. In the presence of the full-length (11-mer) wild-type peptide, the GG motif mutants exhibited the catalytic activity. The observed activity was dependent on the concentration of the peptide, based on which the $K_d$ value for the interaction was determined as $0.15 \pm 0.04$ mM (Figure 63c). The maximum activity was observed at peptide concentrations higher than 0.3 mM, and was comparable to the activity of wt MoaA. Further detailed characterization of the interaction between the peptide variants and MoaA with mutations at potential sites of interaction provided strong evidence for the interaction of the C-terminal tail with the
SAM-binding pocket of MoaA. In addition, my studies of the affinity of SAM binding to MoaA variants using anaerobic isothermal titration calorimetry (ITC) in combination with steady state kinetic characterization suggested that the GG motif is essential for the binding and subsequent reductive cleavage of SAM binding.

In summary, these observations suggest the essential role of the GG motif in the binding of SAM, and subsequent radical initiation. Since the mechanism of initiating radical reaction in radical SAM enzymes is an emerging and unsolved question, [201] further studies on the function of the GG motif will provide important insights into the mechanism of radical SAM enzymes in general.
Figure 63: **In Summary – Characterization and Peptide Rescue of MoaA GG Mutant Activity**

(a) Model of *S. aureus* MoaA homodimer and active site, created by overlaying the structures of MoaA in complex with GTP [12], and MoaA in complex with SAM [12]. Disordered C-terminal tail drawn in pink. (b) Activity of MoaA GG-motif mutants in the presence of the 11-mer peptide with amino acid sequence corresponding to the wt-MoaA or G340A-MoaA. The $k_{cat}$ values were determined based on formation of cPMP in the MoaA (3 µM) assay solution containing 10 µM MoaC, 0.3 mM NaCl, 1 mM SAM, 1 mM DTH, 5 mM DTT, 2 mM MgCl₂, 1 mM GTP, and 500 µM of specified peptide. Reactions were triplicated, and error bars represent standard deviation. (c) Activity of G340A-MoaA in the presence of various concentrations of the 11-mer Wt peptide. The solid line is the non-linear fit to Hill equation [192] with $k_{cat} = 0.055$ min⁻¹, $K_D = 151$ µM, and Hill coefficient = 1.86.
6.5. Conclusion

My thesis project focused on the first step of the biosynthesis of a ubiquitous cofactor, Moco. I have discovered a novel biosynthetic intermediate, and delineated the functions of the two biosynthetic enzymes, MoaA and MoaC. Through a diverse combination of mechanistic, structural, and in vivo techniques, my studies of these enzymes have been providing novel insights into this intriguing biochemical reaction.
References


Biography

Bradley Morgan Hover was born on Saturday, May 4th, 1985, and spent most of his childhood living in South Central New York. Having attended Elmira College, Brad graduated summa cum laude and Phi Beta Kappa in 2007 with an Artium Baccalaureus degree in Biochemistry, and minored in physiological psychology. After spending two years conducting government research, Brad began his graduate career in 2009 at Duke University and received his Philosophiae Doctor degree in Biochemistry in December 2014. During his time at Duke, Brad has participated in a number of activities both within and outside the Department of Biochemistry. He has served as the Biochemistry Department Representative to the Graduate Student and Professional Council (GPSC), and continued to serve on Executive Board of GPSC in various leadership roles (Director of University Affairs: 2011-2013, Attorney General: 2013-2014). He has also sat on the Gordon G. Hammes Faculty Teaching Award Selection Committee, and has previously chaired both the Nozaki Memorial Lectureship Series Selection and Departmental New Student Recruitment Committees. Outside of the Department, Brad has volunteered frequently as an event leader and judge at the annual NC Science Olympiad Competitions.

Honors, Awards, and Recognition:

- Duke University’s Department of Biochemistry Service Award, 2014
- Duke Scholar in Infectious Disease, 2012-2013
- Phi Beta Kappa, 2007
- summa cum laude, 2007
- DeWaters Gorman Memorial Prize in Biochemistry, 2006
- Omicron Delta Kappa, Beta Beta Beta, Phi Eta Sigma Honor Societies, 2006
- Sigma Xi, 2005
- CANY Scholastic Recognition Award, 2003

Publications:

- Hover, BM, Yokoyama, K. Protein C-terminus assisted radical initiation by the radical SAM GTP cyclase, MoaA, in molybdenum cofactor biosynthesis. 2014, (In Preparation).


**Selected Posters & Talks:**

• Hover, BM, Tonthat NK, Schumacher MA, Yokoyama, K. *Mechanism of pyranopterin ring formation in molybdenum cofactor biosynthesis.* Department of Biochemistry Retreat. Duke University. October 2014.

• Hover, BM, Yokoyama, K. *Peptide Rescue of Molybdenum Cofactor Deficiency Causing Mutations in the C-terminus of MoaA.* Department of Biochemistry Retreat. Duke University. April 2014.
