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Radical breakthroughs in natural product and cofactor biosynthesis

Kenichi Yokoyama*

Department of Biochemistry, Duke University Medical Center, Durham, NC, 27710

Abstract

The radical SAM (*S*-adenosyl-L-methionine) superfamily is one of the largest group of enzymes with >113,000 annotated sequences¹. Members of this superfamily catalyze the reductive cleavage of SAM using an oxygen sensitive 4Fe-4S cluster to transiently generate 5'-deoxyadenosyl radical (5'-dA•) that is subsequently used to initiate diverse free radical-mediated reactions. Because of the unique reactivity of free radicals, radical SAM enzymes frequently catalyze chemically challenging reactions critical for the biosynthesis of unique structures of cofactors and natural products. In this perspective, I will discuss the impact of characterizing novel functions in radical SAM enzymes on our understanding of biosynthetic pathways and use two recent examples from my own group with particular emphasis on two radical SAM enzymes responsible for carbon skeleton formation during the biosynthesis of a cofactor and natural products.

Graphical Abstract

1. Introduction

Naturally occurring organic small molecules are frequently characterized by unique and complex structures that are critical for their biological functions. In the past decade, many of the biosynthetic pathways for these natural products were found to involve radical *S*-adenosylmethionine (SAM) enzymes¹. These enzymes form one of the largest enzyme superfamilies, and are characterized by their ability to catalyze the reductive cleavage of SAM using oxygen sensitive 4Fe-4S clusters.² Most frequently, these reactions generate a transient 5'-deoxyadenosyl radical species or its equivalent, which abstracts an H-atom from the substrate and initiates free-radical mediated reactions (Fig. 1).

*Corresponding Author: Telephone: (919) 684-8848; ken.yoko@duke.edu.

Radical SAM enzymes catalyze chemically challenging reactions using free radicals in ways distinct from other free radical utilizing enzymes. Before the discovery of the radical SAM enzymes, except for cobalamin-dependent enzymes, most radical-mediated transformations had been thought to be limited to oxygenates which use molecular oxygen as an oxidant. By comparison, the reactions catalyzed by radical SAM enzymes are independent of molecular oxygen and can be either oxidative or non-oxidative reactions, which significantly extends the scope of enzyme-catalyzed radical reactions. Currently, more than 113,000 sequences have been annotated as radical SAM enzymes, and more than 80 distinct radical SAM reactivities have been proposed or demonstrated. In general, radical SAM enzymes catalyze otherwise chemically difficult reactions such as sulfur insertion, oxidative decarboxylation and C-C bond formation at unactivated carbon centers. Thus, they constitute one of the most functionally diverse enzyme superfamilies, and their functions and mechanisms form a novel paradigm in enzymology.

Due to their unique and frequently irreplaceable functions, many radical SAM enzymes catalyze key steps in biological processes. Therefore, understanding the functions of radical SAM enzymes is important to provide scientific foundations to solving medical problems related to these biological processes. Despite their significance and novelty, studies of radical SAM enzymes have been limited due to several factors. These include the technical challenge associated with handling highly oxygen-sensitive enzymes, diverse and unique reactivities, and limited sequence homologies within the superfamily. Also, the functional prediction of radical SAM enzymes requires knowledge and experience in each specific pathway and the reactivity of free radicals in general. We have been particularly interested in radical SAM enzymes that catalyze the key steps in the natural products and cofactor biosynthetic pathways, specifically those responsible for carbon skeleton formation whose functional characterization would significantly promote the understanding of the pathway. Here, using two of the projects in my own group, I discuss how the novel functions and reactivities of select radical SAM enzymes were found, and how such findings have impacted our understanding of the pathways.

2. Molybdenum cofactor biosynthesis

(1) Introduction

The molybdenum cofactor (Moco) is an organometallic cofactor found in many redox enzymes that are essential for organisms' viability.³⁻⁴ In these enzymes, Moco mediates redox chemical reactions and is essential for their catalysis. Unlike many organic enzyme cofactors, Moco cannot be taken up as a nutrient, therefore requiring *de novo* biosynthesis. As a consequence, Moco biosynthetic enzymes have been linked to several human health problems. For example, in humans, genetic mutations in the Moco biosynthetic enzymes cause an inheritable and fatal metabolic disorder termed Moco deficiency (MoCD)⁵. MoCD is characterized by seizures, progressive neurological symptoms, and unusual brain development caused by sulfite accumulation due to the loss of function of Moco-dependent sulfite oxidase. A recent development in experimental therapy involving supplementation with a Moco biosynthetic intermediate has shown some promise with a five-year survival rate of ~30%, as compared to 100% mortality without the treatment⁶. The current challenge

to this therapy appears to be the timely identification of MoCD and initiation of supplementation treatment.

More recently, Moco biosynthesis was shown to be essential for the virulence of bacterial pathogens such as *Mycobacterium tuberculosis* (MTB)⁷. In MTB, many Moco biosynthetic genes have been shown to be essential for virulence, and especially necessary for the development of latent tuberculosis. Intriguingly, MTB harbors multiple copies of the Moco biosynthetic genes with partially overlapping functions, suggesting the critical role of Moco in the physiology of MTB. The pharmacological inhibition of Moco biosynthesis in MTB has been proposed as the mechanism of action for the anti-tuberculosis compound, TCA1⁸, although the exact mechanism by which inhibition of Moco biosynthesis causes antibacterial activity is not well understood.

In all organisms, Moco is biosynthesized from GTP via three major steps (Fig. 2A); (1) rearrangement of GTP into a cyclic pyranopterin monophosphate (cPMP), (2) sulfur insertion, and (3) molybdate insertion. Subsequently, Moco receives target-specific chemical modifications which finely tune the reactivity of the cofactor, and is then inserted into the active site of Moco-dependent enzymes. The transformation of GTP into cPMP is the first committed step of the Moco pathway, includes the formation of the pterin backbone, and is affected in more than 50% of human MoCD patients. Based on early isotope incorporation studies, the Rajagopalan lab proposed a unique mechanism for this transformation in which the C-8 of GTP is inserted between the C-2' and C-3' of the ribose (Fig. 2A)⁹ to form the pterin ring of Moco. This mechanism was distinct from the other previously identified pterin biosynthetic pathways where the C-8 is lost and not incorporated into the pterin ring. Because of this difference, it was assumed that the formation of the Moco pterin ring involved a unique mechanism, and the elucidation of this mechanism has attracted significant interest among researchers in the fields of Moco biosynthesis and enzymology since the mid-1990s.

Studies in the 1990s and early 2000s revealed that in bacteria, this transformation requires two proteins, MoaA and MoaC.⁴ MoaA belongs to the radical SAM superfamily, while MoaC does not show significant sequence or structural similarities to any functionally characterized enzymes. Since radical SAM enzymes generally catalyze chemically difficult reactions, MoaA was assumed to be responsible for the complex rearrangement required for the transformation of GTP into cPMP, and this initial hypothesis was strengthened by structural characterization of the two proteins. X-ray crystal structures of MoaA were solved in the presence of either SAM¹¹ or GTP¹⁵ bound to the active site, and in these structures, two 4Fe-4S clusters were also observed in the active site. The N-terminal cluster is a canonical radical SAM cluster that binds SAM, whereas the C-terminal cluster binds GTP with the N1 position directly interacting with the unique Fe of the cluster. This interaction was proposed to favor tautomerization of guanine to its pyridinol form upon binding into the active site, and this tautomerization may play a key role in the catalytic cycle of MoaA¹⁶. On the other hand, the MoaC structure revealed no similarity to any functionally characterized enzymes¹⁷. While a putative ligand binding site was proposed based on conservation of an amino acid residue, site-directed mutagenesis and complementation assays using an *E. coli moaC* strain, no ligand was identified either biochemically or structurally¹⁷. Based on

these observations, a single step model for the transformation of GTP into cPMP was proposed¹¹ (Fig. 2B). In this model, MoaA and MoaC form a complex, with MoaA serving as the catalytic subunit and being responsible for all the chemical transformations required to form cPMP from GTP, while MoaC is a regulatory subunit essential for the complete function of MoaA. However, no evidence for the formation of a complex between MoaA and MoaC was found. In addition, MoaC was subsequently shown to bind nucleoside triphosphate. Based on these observations, a stepwise model for Moco formation was proposed¹² (Fig. 2C), in which MoaA catalyzes rearrangement of GTP into pyranopterin triphosphate and MoaC forms the cyclic phosphate ring to yield cPMP. In either model, MoaA was considered to be responsible for the rearrangement reaction to construct the pyranopterin ring, and MoaC was thought to have either no catalytic function or to catalyze only the cyclic phosphate formation.

By the year 2011, when we started the project, the pyranopterin triphosphate model had become the predominant view of the field⁴. However, pyranopterin triphosphate had never been characterized chemically or spectroscopically. In addition, this model was inconsistent with the observation by Schindelin and Hänzelmann¹⁵ that the reaction of MoaA with GTP yielded a molecule that could be converted to dimethylpterin (DMPT) upon acid hydrolysis and incubation with 2,3-butanedione. Since pyranopterin triphosphate would not be derivatized to DMPT under the reported conditions, this observation was interpreted as the presence of a formylaminopyrimidinone intermediate (Fig. 2B). Since no data were reported for this observation¹⁵, it was impossible to evaluate the relevance of the observation of DMPT formation to cPMP biosynthesis. Therefore, we started the project with a particular focus on the structure of the putative MoaA product/intermediate.

(2) Functional characterization of MoaA and MoaC

Initially, we focused on characterizing recombinant bacterial MoaA and MoaC¹⁴. In these studies, we found that MoaA catalyzes the conversion of GTP into a molecule that can be chemically derivatized to DMPT, consistent with the Schindelin laboratory's observation. In addition, we found that this compound is dissociable from MoaA and quantitatively converted to cPMP by purified MoaC, demonstrating the relevance of this compound to Moco biosynthesis for the first time.

Isolation of the MoaA product was initially hampered by a loss of the compound during our purification steps. When we studied the stability of the compound, we found that it was particularly sensitive to O₂, as well as acidic pH and temperatures above 20 °C. In addition, the poor catalytic efficiency of MoaA yielded substoichiometric amounts of the product. Two key developments helped overcome these obstacles¹⁴. The first was the development of a large-scale (gram quantity) purification method for MoaA using coexpression with the *suf* operon. Under this condition, and in the presence of a reductant such as β-mercaptoethanol, MoaA can be purified aerobically which allowed us to perform purifications from >50 L of *E. coli* culture at one time. The purified protein was subsequently degassed and reconstituted with its 4Fe-4S clusters under strictly anaerobic conditions to obtain fully functional MoaA. Another development was the use of strictly anaerobic (< 0.1 ppm O₂) conditions for the MoaA reaction and the product purification through the use of an mBraun glove box

temperature controlled at 10 °C, in which the MoaA product is reasonably stable without detectable decomposition for at least 3 hours. Using these techniques, we isolated ~4 μmol (~2 mg) of the spectroscopically > 90% pure MoaA product from an assay containing ~1.6 g of purified MoaA. The purified compound contained a characteristic absorption band at 322 nm, which decayed upon exposure to O₂ to yield an absorption band at 253 nm with a shoulder at 275 nm, a feature similar to that of guanine base.

Structural characterization of the MoaA product was performed through NMR and MS analyses. To facilitate NMR characterization, we prepared uniformly ¹³C labeled MoaA product, and characterized the compound by ¹H and ¹³C detecting NMR methods. Based on the data from both the NMR and MS analyses, we proposed that the MoaA product is the novel cyclic nucleotide, 3',8-cyclo-7,8-dihydro-GTP (3',8-cH₂GTP, Fig. 2D). To eliminate any possibility that 3',8-cH₂GTP is an artifact of purification, we performed in situ ¹³C NMR characterization of the MoaA reaction with uniformly ¹³C-labeled GTP in an anaerobically sealed NMR tube. Under these conditions, formation of 3',8-cH₂GTP was observed concomitant with the consumption of GTP, and 3',8-cH₂GTP was the only detectable product of the MoaA reaction¹⁸ since no other ¹³C-labeled molecules, including pyranopterin triphosphate, were observed in the reaction. In sum, our combined studies revealed that MoaA catalyzes the transformation of GTP into 3',8-cH₂GTP without the formation of any other previously proposed molecules.

Our kinetic characterizations of MoaA- and MoaC-catalyzed reactions provided evidence that 3',8-cH₂GTP was the physiologically relevant biosynthetic intermediate. Purified 3',8-cH₂GTP was converted to cPMP by MoaC with a very low *K_m* value of <60 nM¹⁴, and MOCS1B, a human homolog of MoaC, also catalyzed the conversion of 3',8-cH₂GTP into cPMP with kinetic parameters comparable to those of *S. aureus* MoaC¹⁴. Unlike other Moco biosynthetic intermediates including cPMP, 3',8-cH₂GTP has never been detected in bacterial or human cell cultures^{4, 19}. Therefore cellular concentrations of 3',8-cH₂GTP are unlikely to be higher than 1 μM, and the observed low *K_m* value of MoaC towards 3',8-cH₂GTP is consistent with this presumed low abundance of 3',8-cH₂GTP inside the cells. In addition, the *k_{cat}* value for the MoaC-catalyzed conversion of 3',8-cH₂GTP to cPMP was four folds greater than that of the MoaA-catalyzed conversion of GTP into 3',8-cH₂GTP (0.17 vs 0.043 min⁻¹, respectively)¹⁴. This observation suggests that the MoaA reaction is likely the rate-limiting step of the two, and when both enzymes are present, 3',8-cH₂GTP is kinetically masked and not detectable. Furthermore, 3',8-cH₂GTP apparently exhibits product inhibition of MoaA, and MoaA catalyzes substoichiometric turnover in the absence of MoaC¹⁴. These kinetic behaviors can explain why 3',8-cH₂GTP eluded characterization for the past two decades in both in vitro as well as in vivo analyses.

In sum, the combined evidence suggests that 3',8-cH₂GTP is the physiologically relevant product of MoaA and the substrate of MoaC^{14, 18}. With these discoveries, we proposed revised functions for these two enzymes in the Moco pathway^{14, 18}, whereby MoaA catalyzes the conversion of GTP into the oxygen-labile cryptic biosynthetic intermediate, 3',8-cH₂GTP, which is then converted to cPMP by a complex rearrangement reaction catalyzed by MoaC (Fig. 2D).

Our proposal was distinct from all previous hypotheses on Moco biosynthesis because the formation of the pterin ring had long been assumed to be catalyzed by MoaA with minimal input from MoaC, while we found that MoaC is actually the enzyme responsible for pterin ring formation. Therefore, our proposal was met with surprise in the field. However, in retrospect, all of the previous observations for the MoaA product reported by other groups are also consistent with 3',8-cH₂GTP. The formation of a compound with acid-labile triaminopyrimidinone base in the MoaA reaction first reported by Schindelin and Hänzelmann¹⁵, and reproduced by us¹⁴, is consistent with 3',8-cH₂GTP, and not with pyranopterin triphosphate. Second, the Begley lab reported that the MoaA product has an absorption band at ~320 nm¹³, which is consistent with 3',8-cH₂GTP. While the Begley lab interpreted this absorption feature as pyranopterin triphosphate, λ_{max} values of reported pyranopterin compounds do not exceed 300 nm²⁰. Finally, while the formation of an oxidized pterin was reported when MoaA reaction products were treated with KI and I₂²¹, the amount of this putative oxidized pterin was less than 3% of 3',8-cH₂GTP, and the rate of its formation was too slow to be relevant for cPMP formation¹⁸. Therefore, all of the earlier observations are consistent with 3',8-cH₂GTP being the MoaA product, suggesting that this compound had been previously detected, but its structure could not be accurately assigned without its isolation. While the isolation of 3',8-cH₂GTP requires rigorous anaerobic experiments, our newly developed in situ ¹³C NMR method¹⁸ provides a facile approach to determine the identity of the MoaA product. Thus, this method can be used to characterize MoaA from other organisms by any labs interested in the function of MoaA.

(3) Catalytic mechanism of MoaC

Our discovery of 3',8-cH₂GTP revealed a novel function for MoaC to catalyze the complex rearrangement required for the conversion of 3',8-cH₂GTP to cPMP. Many enzymes have been found to catalyze multi-step rearrangement reactions, particularly in biosynthetic pathways for cofactors such as flavin, thiamine, and pyridoxamine²². While the chemical mechanism of such enzymes has been extensively explored, it is still largely unknown how enzymes control the reactivity of unstable intermediates and guide their complex reactions to yield only one intended product.

To obtain further insights into the mechanism of MoaC catalysis, we determined X-ray crystal structures of MoaC in complex with its substrate¹⁸. Since our initial crystallization efforts using wt-MoaC yielded only the structure in complex with cPMP, we also looked at MoaC variants with significantly reduced activity. Such variants were identified through an alanine scanning of conserved acidic and basic residues that revealed six amino acid residues essential for catalytic function. Among these variants, K51A-MoaC yielded a structure in complex with the MoaA product where the ligand electron density at the interface of each dimer unit of the hexameric K51A-MoaC was most consistent with our proposed structure of 3',8-cH₂GTP (Fig. 3A), and was not consistent with other previously proposed MoaA products or reaction intermediates, including pyranopterin triphosphate. Furthermore, in our wt-MoaC crystal structures in complex with cPMP, cPMP was found in the pocket identical to the 3',8-cH₂GTP binding site (Fig. 3B), and this ligand binding pocket consists of the catalytically essential six amino acid residues identified by our alanine scanning. These

observations together suggest that this pocket is the active site of this enzyme, and provided further evidence for 3',8-cH₂GTP being the physiologically relevant substrate for MoaC.

The structures also provided insights into the mechanism of the MoaC-catalyzed complex rearrangement reaction¹⁸. The structure of MoaC in complex with 3',8-cH₂GTP and that with cPMP exhibited distinct conformation of the loops around the active site. In the wt-MoaC complex with cPMP, K51 forms a salt bridge with D128, and as a consequence, the loop 3 that harbors K51 is closed and forming an extended H-bond interaction with the N-terminal loop (Fig. 3B). On the other hand, in the K51A-MoaC•3',8-cH₂GTP complex structure, loop 3 is in an open conformation, and the space occupied by K51 in the wt-MoaC•cPMP structure is now occupied by the 2'-OH of 3',8-cH₂GTP (Fig. 3A). With loop 3 in the open conformation, the H-bond interaction between loop 3 and the N-terminal loop is no longer possible, leaving the N-terminal loop disordered and not detectable in the crystal structure. With these observations, we hypothesized that loop dynamics are a part of the catalytic mechanism (Fig. 3C). In this model, the resting state of MoaA exists in the two conformations, and only the open conformation binds 3',8-cH₂GTP and initiates the early steps of the catalysis. This transformation yields an intermediate with the ribose ring conformationally flexible around 2'-OH, which allows the K51 residue to directly interact with D128. The closure of loop 3 also facilitates the closure of the N-terminal loop through the backbone H-bond interaction. This movement of the N-terminal loop triggers the relocation of the triphosphate moiety of 3',8-cH₂GTP for the subsequent cyclic phosphate formation and production of cPMP.

This conformationally guided model predicted the presence of an intermediate in the K51A-MoaC mutant. Therefore, we reinvestigated the reaction products of active site mutants of MoaC using ion-pairing HPLC under anaerobic conditions and found that the two active-site variants K51A and K131A produce an on-pathway reaction intermediate X that was converted to cPMP when incubated with wt-MoaC¹⁸. Characterization of this intermediate by chemical derivatization suggested that it is a nucleoside triphosphate with an acid-labile triaminopyrimidinone base. The UV-vis absorption spectrum of this compound had a λ_{max} at 270 nm and was comparable to the reported spectrum for triaminopyrimidinone nucleotides. Furthermore, MS analysis revealed the molecular weight to be 18 Da greater than that of 3',8-cH₂GTP. Together, these observations suggested that 3',8-cH₂GTP had undergone a hydration or hydrolysis reaction in the active site resulting in intramolecular cleavage within the dihydroguanine moiety to yield a triaminopyrimidinone functionality.

The timing of the cyclic phosphate formation was investigated using 3',8-cH₂GMP[CH₂]PP, a noncleavable analog of 3',8-cH₂GTP with a methylene in place of the oxygen between the α and β phosphates²³. The reaction of 3',8-cH₂GMP[CH₂]PP with MoaC did not yield any diffusible products, but instead caused irreversible inhibition of MoaC. Chemical analysis of MoaC treated with 3',8-cH₂GMP[CH₂]PP revealed a dead-end product (compound Y) that tightly bound to MoaC, harbored an acid-labile triaminopyrimidinone base, but did not have a pyranopterin structure. MS analysis of this MoaC•compound Y complex suggested that compound Y was covalently linked to MoaC and that modification has a molecular weight 18 Da smaller than 3',8-cH₂GMP[CH₂]PP. The location and the relevance of the covalent bond to the MoaC catalysis are currently unknown, but the covalent bond is likely a

consequence of the accumulation of a highly electrophilic intermediate that could react with nucleophilic residues in the active site. These observations suggest that the formation of the pyranopterin structure is coupled to cyclic phosphate ring formation⁴ and that the immediate precursor to this ring formation is a highly electrophilic species. They also suggest that pyranopterin triphosphate, the previously proposed intermediate of cPMP formation, is unlikely to exist as an intermediate.

Based on these structural and biochemical observations, we proposed the first detailed working hypothesis for MoaC catalysis (Fig. 3D)¹⁸. The key aspect of this model is that the conformational flexibility of the two loops allows the introduction of general acid/base catalysts in response to the change in the shape of reaction intermediates. The thermodynamic stabilities of the loop conformations may play important roles in stabilizing and orienting different intermediates during the course of the reaction to promote the multi-step rearrangements towards the product. In this model, MoaC initiates the catalysis by a cleavage of the C8-N9 bond by polarizing the C6=O carbonyl bond through the H-bond interaction with the backbone amides of E112 and M113 and a dipole moment of the α -helix containing E112 and M113. From the resulting iminium intermediate, two mechanisms are conceivable. In mechanism A, the iminium is hydrolyzed to an aldehyde intermediate X. In mechanism B, the C2'-C3' bond is cleaved by a retro-aldol reaction using D128 as a general base catalyst, followed by isomerization and hydrolysis of the second iminium intermediate to yield an aldehyde/enol intermediate X. The former is more likely in solution chemistry, while the latter explains the absence of intermediate X formation in the D128A-MoaC assay. In either case, the intermediate X should be more conformationally flexible around C2', which would allow the closure of loop 3 and incorporation of K51 into the active site. This movement is likely driven by the formation of H-bond interactions between K51 and D128.

The K51 residue as well as the catalytically essential K131 would serve as general acid/base catalysts during the subsequent retro-aldol/aldol type ring expansion reaction to yield the hexose intermediate. The roles of K51 and K131 are based on the accumulation of the intermediate X in MoaC variants lacking these residues. The hexose intermediate would then undergo dehydration to yield an α,β -unsaturated ketone intermediate, which we propose to be the immediate precursor of pyranopterin ring formation. We also propose that this pyranopterin ring formation proceeds in concert with cyclic phosphate ring formation and that this reaction is facilitated by a closure of the N-terminal loop. This closure is likely thermodynamically favored when it is coupled to the loop 3 closure because the closed forms of the loops are stabilized by the formation of backbone H-bond interactions. This thermodynamic energy is likely used to replace the triphosphate H-bonding partner of H77 with the backbone carbonyl of V16 in the N-terminal loop and to relocate the triphosphate for interaction with E112. Once the α -phosphate is positioned close enough to the O4 carbonyl oxygen, the 5-NH₂ attacks the α,β -unsaturated ketone to initiate the concerted formation of the pyranopterin and cyclic phosphate rings. This step involves irreversible cleavage of pyrophosphate and likely provides a thermodynamic driving force for the overall reaction.

Our proposal for the MoaC catalysis provides a testable model to investigate the roles of protein dynamics in enzyme-catalyzed multi-step rearrangement reactions. In this context, there are several key questions that need to be addressed including the structures of the trapped intermediates, the site of covalent modification with compound Y, and the exact timing of the closures of the two loops. It is interesting to understand if and how the thermodynamic stabilities of the loop conformations contribute to the progress of the complex rearrangement reaction. Addressing these questions in this system will provide insights into the role of protein conformational dynamics in the types of enzyme-catalyzed multi-step rearrangement reactions frequently observed in natural product and cofactor biosynthesis.

(4) Mechanism of MoaA catalysis and MoCD

MoaA is one of the first radical SAM enzymes characterized to be responsible for C-C bond formation, which is emerging in the past 5–6 years. In humans, point mutations in the MoaA homolog MOCS1A have been found to cause MoCD, but how these point mutations cause MoCD is, however, largely unknown. Therefore, an additional point of focus for us is to determine the mechanism by which point mutations found in human MoCD patients affect the function of MOCS1A from an enzymological perspective. Such studies would be expected to provide insight into not only MoCD but also into mechanistic questions being asked in the radical SAM enzymology field in general.

Our proposed mechanism for MoaA catalysis is shown in Fig. 4A. In this mechanism, MoaA catalyzes abstraction of the GTP H-3' atom using the 5'-deoxyadenosyl radical, and the position of H-atom abstraction has been demonstrated using deuterated GTP^{13–14}. In the crystal structures, C5' of SAM is positioned close to the C3' of GTP^{11, 15}, consistent with the H-3' abstraction mechanism (Fig. 4B). The resulting C3' radical then attacks C8 of the guanine base to form an aminyl radical intermediate that is subsequently reduced by transfer of both an electron and a proton to yield 3',8-cH₂GTP. The exact mechanism of the aminyl radical reduction, including the source of the electron and the proton, is currently unknown, although the C-terminal 4Fe-4S cluster has been proposed to serve as a Lewis acid¹⁶ or electron donor¹⁴.

The reported MoCD-causing mutations within MOCS1A are clustered in three locations⁵. The first is the Cys ligands of the two 4Fe-4S clusters, and these mutations result in loss of the catalytically essential 4Fe-4S clusters. The second location is the conserved three Arg residues (R17, R266, and R268; numberings based on *S. aureus* MoaA) in the active site. R266 and R268 are within H-bonding distance of the O6 and may be involved in shifting the keto-enol tautomerization of the N1-O6 moiety proposed to play a role in the catalysis, potentially during the aminyl radical reduction. R17 is within H-bonding distance of both the α - and β -phosphate as well as N7 of the guanine base, but its function is unknown.

The third location is the two Gly residues at the C-terminal end (G339 and G340). These two Gly residues (GG-motif) are strictly conserved in MoaA/MOCS1A homologs from all organisms except for those in archaea. In the crystal structures of MoaA, the C-terminal 11 amino acid residues, including the GG motif, are disordered. Therefore, the location and the mechanism by which mutations in the GG motif cause MoCD is unknown. Hänzelmann et

al. characterized the GG-motif mutants using an *E. coli moaA* strain harboring a plasmid coding human MOCS1A mutants with mutations in the GG-motif²⁴. Their study revealed that smaller substitutions, such as Ala, are permissive, while sterically bulkier substitutions (e.g. Val, Asp, and Pro) of the conserved glycines completely abolished activity evaluated based on the restoration of Moco biosynthesis. Since the strength of the plasmid promotor affected the activity measured for some of the variants, the observations suggested a variable level of activity loss depending on the substituting amino acids. These observations were reminiscent of the GG-motif found in ubiquitin and the small subunit of MPT synthase (MoaD). In these proteins, the C-terminal carboxylate is adenylated for subsequent modification by E1 ubiquitin ligase or sulfide, respectively, and the small size of the two Gly residues at the C-terminus is critical for insertion into the active site of the adenylating enzyme²⁵. While adenylation is unlikely for MOCS1A, the GG-motif could still be important for its interaction with the active site of MOCS1A or MOCS1B.

To obtain insights into the function of the human MOCS1A GG-motif, we used *S. aureus* MoaA as a model²⁶. Our in vitro characterization of purified MoaA variants suggested that even the smallest perturbation made by replacing Gly with Ala completely abolished the activity of the enzyme below the detection limit (0.4% of wt activity), but no effects of the mutations on the Fe-S cluster loading or quaternary structure were detectable. Intriguingly, we found that the activity of the GG-motif variants of *S. aureus* MoaA was restored by a synthetic 11-mer peptide with an amino acid sequence corresponding to the C-terminal tail of wt-MoaA (RKKINMNYIGG)²⁶. This observation suggested that the GG-motif mutants of MoaA might have the complete function except for the C-terminal tail.

We also found that restoration of the GG-motif mutant activity was dependent on the sequence of the peptide²⁶. The GG-motif in the peptide was essential for the restoration, whereas the removal of the three cationic residues at the N-terminus of the peptide (RKK) reduced the restored activity by ~10 fold. A similar reduction in the activity was observed by mutating the RKK residues in MoaA, suggesting that the 11-mer peptide is acting as a surrogate of the C-terminal tail of wt-MoaA. The 11-mer peptide exhibited a K_d of 151 μ M and a positive cooperativity with a Hill coefficient of 1.9, suggesting that the binding of the peptide to one of the two subunits of the MoaA dimer may facilitate binding of the peptide to the other subunit. Steady-state kinetic characterization and isothermal calorimetric (ITC) titration experiments suggested that SAM binding was significantly perturbed in G340A-MoaA ($K_m = 5.1$ vs 37 μ M, K_d , 1.7 vs > 90 μ M), whereas the GTP binding was not ($K_m = 3.1$ vs 7.7 μ M, K_d , 5.1 vs 5.2 μ M)²⁶. Furthermore, Ala scanning of acidic residues on the surface of G340A-MoaA suggested that the RKK residues on the peptide interact with D198 of MoaA located adjacent to the SAM-binding site²⁶. In the crystal structures, the active site of MoaA is open and exposed to solvent^{11, 15}, which is a sharp contrast to other radical SAM enzymes, suggesting that a significant portion of the active site is missing in the reported crystal structures. Together with our observations, it seems most likely that the C-terminal tail forms a part of the active site essential for SAM binding, and the small size of the GG-motif is likely important for the C-terminal tail to fit into the active site.

Because the C-terminal tail of MoaA is essential for SAM binding, it could also be involved in SAM cleavage and/or H3'-atom abstraction. When we studied the reaction of G340A-

MoaA in the presence of the wild type 11-mer peptide (G340A-MoaA•11-mer) using [3'-²H]GTP as the substrate, a significant increase in the kinetic isotope effect (KIE) was observed relative to that for wt-MoaA (KIE = 3.0 vs 1.3)²⁶. This observation suggests that H-3' atom abstraction is partially rate determining in the G340A-MoaA•11-mer reaction. In the wt-MoaA reaction, this step is kinetically masked by a preceding rate-determining step. Importantly, the formation of 5'-dA and 3',8-cH₂GTP in the G340A-MoaA•11-mer reaction was very well coupled, and no significant amount of abortive cleavage of SAM was observed. This observation may be explained by either (1) a slower H-3' atom abstraction due to a perturbation in the distance between C5' of the transient 5'-dA• and H-3', or (2) an altered kinetics of the steps preceding the H-3' atom abstraction. Regardless of the cause, the observed change in the primary KIE suggests kinetic perturbations in the radical initiation step in the G340A-MoaA•11-mer reaction.

This observation is particularly intriguing regarding understanding the mechanism of SAM cleavage in radical SAM enzymes in general. While the predominant view of the field is that the C5'-S bond of SAM is cleaved homolytically by a single electron transfer from the canonical [4Fe-4S]⁺ cluster (Fig. 5, top), such a mechanism would make SAM cleavage thermodynamically unfavorable by ~390 mV²⁷. In addition, the characterization of Dph2²⁸, which cleaves the C₈-S bond of SAM to generate a 3-amino-3-carboxylpropyl radical, suggests that enzymatic SAM cleavage could take place either on the C5'-S bond or the C₈-S bond. This regio-specificity of SAM cleavage has to be taken into consideration when proposing a mechanism for SAM cleavage²⁹. Recently, the Broderick and Hoffman labs reported a novel organometallic species with the C5' of 5'-dA covalently connected to one of the Fe of the 4Fe-4S cluster in pyruvate formate lyase activating enzyme (PFL-AE)³⁰. While it is still unknown if this species is a reaction intermediate preceding the 5'-dA• formation or a species formed by the reaction of 5'-dA• with the [4Fe-4S]²⁺ cluster, it raised the possibility that SAM cleavage may proceed by a heterolytic mechanism through a nucleophilic attack of the unique Fe of 4Fe-4S cluster on the C5' of SAM (Fig. 5, bottom). While the nucleophilicity of the unique Fe remains unknown, such a mechanism would avoid the thermodynamically unfavorable homolytic cleavage and explain the regio-specificity of SAM cleavage. In the G340A-MoaA•11-mer reaction, the change in the primary KIE revealed kinetic perturbations at the radical initiation step. Thus, further characterization of this reaction may provide mechanistic and/or structural insights into the SAM cleavage, and the detailed kinetic and spectroscopic characterization of this reaction is currently underway.

3. Antifungal peptidyl nucleoside natural product biosynthesis

The incidence of invasive fungal infections has been emerging in the past two decades due to a combination of several factors including increasing travel in and out of endemic regions, climate change, and an increasing number of immunocompromised patients.³¹ However, the current clinical antifungal arsenal has been limited due to the toxic side effects of, and emerging resistance against, existing antifungal drugs. Unfortunately, the development of new antifungal agents has been hampered by the challenge of selective killing of the eukaryotic pathogens without toxicity to humans. Under such circumstances, natural products that inhibit fungal cell wall biosynthetic enzymes are unique and highly promising

candidates for clinical development, as they exhibit potent and highly selective antifungal activities. This unique activity is due to the fact that the cell wall (Fig. 6A) is essential for the viability of fungi and absent in humans. Among such natural products, the peptidyl nucleoside (PN) antifungals, represented by nikkomycins and polyoxins (Fig. 6B), exhibit antifungal activities through inhibition of chitin synthase, an enzyme required for the formation of chitin in the fungal cell wall (Fig. 6A). Because of their potency and selectivity, PNs have been used for agricultural purposes, and are currently under clinical investigation for human use.

PNs are structurally characterized by the presence of the C5'-modified nucleoside, aminohexuronic acid (AHA), which mimics the uridine moiety of UDP-GlcNAc, the substrate for chitin synthase, and therefore is critical for the antifungal activity. AHA is biosynthesized from UMP and phosphoenol pyruvate (PEP) via the C5'-extended high-carbon nucleoside, octosyl acid (OA, Fig. 6B). While the conjugation of PEP and UMP to yield enolpyruvyl UMP (EP-UMP) was shown to be catalyzed by NikO and PolA in the nikkomycins and polyoxin pathways, respectively³²⁻³³, the mechanism by which OA is formed from EP-UMP was unknown.

The characteristic bicyclic structure of OA has also been found in other antifungal nucleoside natural products such as ezomycins and malayamycins (Fig. 6B). The key step in OA biosynthesis is the formation of the C5'-C6' bond, which was originally proposed to proceed through oxidation of the EP-UMP C-5' to an aldehyde, followed by its conversion to 7'-hydroxy octosyl acid (Fig. 6C).³² This model was based on the precedent set by biosynthesis of the caprazamycin-class of antibacterial nucleosides,³⁴ and the identification of two conserved α -ketoglutarate (α -KG) dependent dioxygenase genes in the nikkomycin and polyoxin biosynthetic gene clusters (*polD* and *polK* in polyoxin).³² However, no experimental evidence was available; the proposed intermediates were all hypothetical compounds that have never been detected experimentally, and the catalytic functions of the putative α -KG dependent dioxygenases have never been demonstrated. In addition, the transformation of the aldehyde to 7'-hydroxy octosyl acid is chemically challenging considering the poor nucleophilicity of C3''. In fact, when we tested the activity of recombinant PolD and PolK, the enzymes did not oxidize any of the nucleoside/nucleotides we tested even though the purified enzymes contained the non-heme iron center and were able to oxidize α -KG into succinate.

Based on these observations and considerations, we sought for an alternative mechanism for the OA biosynthesis. For formation of the C5'-C6' bond of octosyl acid, the C5' of EP-UMP has to be activated, likely by an oxidative mechanism. The only oxidative enzyme conserved among the PN biosynthetic gene clusters, other than the two putative α -KG dependent dioxygenases (PolD and PolK), is PolH, a putative radical SAM enzyme. Although PolH does not exhibit significant sequence similarities to functionally characterized radical SAM enzymes, it is chemically feasible for a radical SAM enzyme to catalyze either the oxidation of C5' of EP-UMP or even cyclization to form the bicyclic structure of OA.

To determine whether PolH might be involved in OA formation, we prepared recombinant PolH. It appeared to be even more oxygen sensitive than MoaA since aerobically expressed or purified PolH could not be loaded with 4Fe-4S clusters even after chemical reconstitution, and the purified PolH precipitated even in the presence of low concentrations of oxygen (~10 ppm). This is a very good demonstration of how the extent of oxygen sensitivity varies significantly among radical SAM enzymes. When PolH prepared and reconstituted under strictly anaerobic conditions was tested for its activity with various uridine nucleotides and nucleosides, only EP-UMP gave a product. Isolation and structural characterization of the product identified it as octosyl acid 5'-phosphate (OAP, Fig. 6B), demonstrating that PolH alone is sufficient to convert EP-UMP into OAP³⁵. An essentially identical observation was made for NikJ, the PolH homolog in the biosynthesis of nikkomycins³⁵. When the two enzymes were characterized by steady-state kinetic analysis, their catalytic efficiencies were comparable to that of PolA/NikO, indicating the physiological relevance of the reconstituted activity³⁵. The Chen and Xiao groups later reported the identical function for PolH³⁶, in which they prepared *polH* gene knockout strain of the polyoxin producer, *S. cacaoi*, and demonstrated that the *polH* gene is essential for polyoxin biosynthesis, and the mutant strain accumulated 5'-enolpyruvyl uridine, a shunt metabolite presumably derived from EP-UMP. Together, these observations from two independent groups established that the unique bicyclic structure of octosyl acid is constructed from EP-UMP by PolH/NikJ.

Functional characterization of PolH revealed that it is a member of the emerging group of radical SAM enzymes that catalyze C-C bond formation. Most of these enzymes catalyze key steps in pathways responsible for the formation of the carbon backbone of the metabolites, and the common mechanism of these enzymes is attack of a carbon-centered radical on an sp^2 center. The resulting radical intermediate is then quenched reductively or oxidatively. While radical quenching is an essential step in all the C-C bond forming radical SAM enzymes, little to no evidence is available for their mechanisms. For example, as discussed above, MoaA catalyzes C-C bond formation that requires an oxidative quenching of the aminyl radical intermediate (Fig. 4A). While the C-terminal 4Fe-4S cluster was proposed as a reductant¹⁴, no evidence is available. In fact, the C-terminal cluster was also proposed to serve as a Lewis acid to shift the keto-enol tautomerization of the N1-O6 moiety¹⁶. Much less is known for other C-C bond forming radical SAM enzymes. Therefore, we have focused on the elucidation of the mechanism of radical quenching during PolH catalysis.

In PolH, catalysis is most likely initiated by abstraction of the EP-UMP H-5' by 5'-dA• (Fig. 7A), followed by an attack of the C5' radical on C3''' of the enolpyruvyl moiety to form a C7' radical intermediate. This radical intermediate must then be reduced by transfer of both an electron and a proton. Based on the incorporation of solvent deuterium into the 7' position of OAP, we hypothesized that C7' radical reduction is catalyzed by a redox-active Cys or Tyr residue in PolH. To identify that residue, we performed Ala scanning of Cys and Tyr residues conserved among homologs of PolH and NikJ. When C209 was mutated, the enzyme produced a mixture of two products, OAP, and its C7' epimer³⁵. Further EPR characterization of the C209S mutant revealed the accumulation of a C7'-centered radical intermediate³⁵. These observations suggested that the reaction proceeds through stereospecific quenching of the C7'-radical intermediate by the redox-active C209 residue

(Fig. 7A). While it is currently unknown how the resulting thiyl radical is reduced back to thiol, conserved Tyr residues expected to be close to the active site in our homology model of PolH³⁵ may be responsible for shuttling the radical to the protein surface where dithionite or other physiological reductants are available to serve as the terminal electron donor. Further structural and enzymological characterization of NikJ is currently underway in my laboratory to address these mechanistic questions. In sum, our studies on NikJ and PolH provide the first evidence for a mechanism of radical control in C-C bond forming radical SAM enzymes and demonstrate the significance of a radical quenching mechanism to the product of the reaction.

Our characterization of PolH and NikJ also revealed that the mechanism of carbon extension at C-5' for the antifungal PNs is distinct from those reported for several different classes of antibacterial nucleoside natural products^{34, 37}, where C5' extension proceeds via oxidation of UMP C-5' to an aldehyde followed by an aldol condensation-type two-electron mechanism. Considering the unique structure, it is highly likely that other antifungal nucleosides with the OA structure are biosynthesized by homologs of NikJ/PolH. To test such possibility, we searched the genome sequence databases for homologs of NikJ and NikO³⁵, the two enzymes essential for the formation of OAP from UMP and PEP. NikJ homologs were defined based on the conservation of the catalytically essential C209 residue. As a result, we found more than 25 operons that contain homologs of NikJ and NikO, and other putative nucleoside modifying enzymes, including a putative pseudouridine synthase (Fig. 7B)³⁵. These analyses suggest that nucleoside natural products containing an OA moiety are likely more prevalent than is currently thought and that the combination of the *nikO* and *nikJ* genes could serve as a genetic marker to identify biosynthetic gene clusters for these nucleoside natural products, possibly leading to the identification of new antifungals.

4. Conclusion

Our studies on the biosynthesis of Moco and antifungal PNs demonstrate the impact of functional and mechanistic characterization of radical SAM enzymes on medically important pathways. Further examples of the impactful characterizations of radical SAM enzymes are emerging from other groups. For example, studies on homologs of ThiC, the enzyme responsible for complex rearrangement reactions during thiamine biosynthesis, revealed the existence of radical SAM enzymes responsible for the biosynthesis of the lower ligand of cobalamine in anaerobic bacteria³⁸, while work on a highly underexplored class C radical SAM methylase led to the discovery of a novel mechanism in anaerobic heme degradation in pathogenic *E. coli*³⁹. Considering the abundance of radical SAM enzymes and their highly underexplored nature, many more breakthrough findings are likely to be made through functional and mechanistic characterization of radical SAM enzymes. Therefore, further studies on radical SAM enzymes are not only the interests of enzymologists but are also important to understand various metabolic pathways.

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ABBREVIATIONS

Moco	Molybdenum cofactor
MoCD	Moco deficiency
GTP	guanosine 5'-triphosphate
3',8-cH₂GTP	3',8-cyclic-7,8-dihydro-GTP
cPMP	cyclic pyranopterin monophosphate
GMP[CH₂]PP	guanosine [(α , β)-methyleno]triphosphate
PN	peptidyl nucleoside
AHA	aminohexuronic acid
PEP	phosphoenol pyruvate
EP-UMP	3'-enolpyruvyl UMP
OA	octosyl acid
OAP	octosyl acid 5'-phosphate
α-KG	α -ketoglutarate
UDP-GlcNAc	uridine 5'-diphospho- <i>N</i> -acetylglucosamine

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Figure 1.
Reductive cleavage of SAM reaction common to all radical SAM enzymes.

Figure 2.

Moco pathway and proposed functions of MoaA and MoaC. (A) Overview of the Moco biosynthetic pathway. The symbols on GTP and cPMP indicate the source of the carbon and nitrogen atoms in cPMP as determined by isotope labeling studies^{9–10}. (B and C) Previously proposed functions of MoaA and MoaC with a single step mechanism¹¹ (B) and stepwise mechanism^{12–13} (C). (D) Our proposal using 3',8-cH₂GTP as the product of MoaA and substrate for MoaC¹⁴.

Figure 3.

MoaC-catalyzed conversion of 3',8-CH₂GTP to cPMP. (A and B) X-ray crystal structures of K51A-MoaC in complex with the substrate, 3',8-CH₂GTP (A), and wt-MoaC in complex with the product, cPMP (B). (C) The proposed motion of loop 3 and the N-terminal loop during MoaC catalysis. (D) Proposed conformationally guided mechanism of MoaC catalysis. Figures were adapted with permission from *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 6347-52.

Figure 4. MoaA-catalyzed conversion of GTP to 3',8-cH₂GTP. (A) The proposed mechanism of MoaA catalysis. The transfer of the H atom at the 3' position to the 5' position of 5'-dA was demonstrated using deuterium-labeled GTP¹³⁻¹⁴. (B) The active site structure of MoaA. SAM was modeled based on the comparison of the crystal structures of MoaA in complex with SAM (PDB ID, 1TV8) and that in complex with GTP (PDB ID, 2FB3). Reprinted with permission from *J. Am. Chem. Soc.* **2013**, *135*, 7019-32. Copyright 2013 American Chemical Society.

Figure 5.
Possible mechanisms for SAM cleavage by radical SAM enzymes.

Figure 6. (A) Fungal cell wall structure. (B) Putative biosynthetic pathways for OA-related antifungal nucleosides. (C) Previously proposed mechanism for OA biosynthesis.

Figure 7.

(A) Proposed mechanism for PolH catalysis. (B) Putative antifungal nucleoside biosynthetic gene clusters.