Published in final edited form as:

Biochim Biophys Acta. 2015 September ; 1854(9): 1073–1077. doi:10.1016/j.bbapap.2015.04.008.

Molybdopterin Biosynthesis – Mechanistic studies on a novel MoaA catalyzed insertion of a purine carbon into the ribose of GTP

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Graphical Abstract

Introduction

Molybdenum is a required nutrient for plants, animals and microorganisms and is involved in many redox reactions implicated in the global carbon, sulfur, and nitrogen cycles.^{1,2} With the exception of nitrogenase, all molybdenum-requiring enzymes use molybdopterin as the metal binding ligand. Nitrate reductase, sulfite oxidase, xanthine dehydrogenase and aldehyde oxidase are well known molybdenum cofactor dependent enzymes. In humans, mutations in the molybdopterin biosynthetic genes lead to death within months of birth.³ Molybdopterin biosynthetic studies have played a critical role in developing the right supplement to overcome this genetic deficiency.⁴ While the biosynthesis of molybdopterin has been extensively studied, the chemical details of the first step, involving a remarkable radical SAM enzyme-catalyzed rearrangement of GTP, remains to be elucidated. Recent progress on the mechanism of this reaction will be described in this paper.⁵

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Biosynthetic pathway and labeling studies

The biosynthesis of molybdopterin is similar in plants, animals and microorganisms. The biosynthetic pathway is shown in Figure 1A. The early steps, catalyzed by MoaA and MoaC, use guanosine-5'-triphosphate (GTP **1**) to synthesize cyclic pyranopterin monophosphate (cPMP **2**). The next steps involve the thiolation of the molybdopterin to form **3** followed by ATP-dependent metal incorporation to give **5**.⁵

Labeling studies have established that the MoaA-MoaC catalyzed conversion of GTP **1** to cPMP **2** involves the insertion of the C8 of GTP between the C2' and C3' carbons as shown in Figure 1B.^{6,7} To the best of our knowledge, this ribose carbon-insertion chemistry is a novel transformation in carbohydrate chemistry.

Structural studies on MoaA

Prior to the mechanistic studies, a crystal structure was available showing that MoaA is a member of the radical S-adenosyl methionine (SAM) superfamily of enzymes and harbors two [4Fe-4S] clusters. The N-terminal [4Fe-4S] cluster was responsible for the reductive cleavage of SAM to the 5'-deoxyadenosyl (5'-dA) radical and L-methionine and the C-terminal [4Fe-4S] cluster was proposed to bind GTP **1** (Figure 2).⁸⁻¹⁰ Subsequent EPR studies suggested that N1 of GTP rather than the amino group binds to the cluster.¹⁰

Reconstitution of MoaA

The first steps in the molybdopterin biosynthetic pathway involve the MoaAMoaC catalyzed conversion of GTP **1** to cPMP **2** (Figure 1B). However, the product of MoaA was previously unknown. Our *in vitro* reconstitution of MoaA confirmed that MoaA was a radical SAM enzyme and demonstrated that it catalyzed the conversion of SAM and GTP to 5'-dA and pyranopterin triphosphate (PTP; **6**) respectively (Figure 3).¹¹ PTP **6** is highly oxygen-sensitive, therefore prior to HPLC purification, it was treated with phosphatase and oxidized with KI/I₂ to form **7** as a stable product which was characterized by NMR and LCMS analysis. It was also trapped as oxime **8** using o-(pentafluorobenzyl) hydroxylamine (PFBHA) (Figure 3). To rule out the possibility of MoaC contamination leading to the formation of PTP **6**, MoaA was overexpressed and purified from an *E. coli* (MoaC⁻) strain. The isolated protein showed the same reactivity, confirming the formation of PTP **6** as the product of MoaA.¹¹

Identification of the hydrogen atom abstracted by the 5'-dA radical

Site-specifically deuterated isotopomers of GTP were used to identify the hydrogen atom transferred to the 5'-dA radical in the MoaA-catalyzed reaction. LCMS analysis of the 5'-dA generated using universally labeled GTP (i.e. deuterium on all non-exchangeable sites) revealed an increase of a single mass unit ([M+H] - 253.1Da) demonstrating that only one of the deuterium atoms from the substrate was incorporated into 5'-dA. No deuterium incorporation was observed with 8-²H-GTP demonstrating that the deuterium was abstracted from the ribosyl part of GTP. No deuterium incorporation was observed with 2'-²H-GTP and a single deuterium incorporation was observed with 3',4',5',5'-²H₄-GTP. This further localized the site of deuterium incorporation to C3', C4' or C5' of the ribose. Final

localization of the hydrogen atom transfer was achieved by demonstrating a single deuterium transfer from 3'-²H-GTP to 5'-dA. This demonstrated that the 5'-dA radical abstracts a hydrogen atom from the 3' carbon of GTP.¹²

Mechanistic proposal for MoaA catalyzed reaction

A mechanistic proposal for the MoaA-catalyzed reaction is shown in Figure 4.¹¹ In this proposal, the 5'-dA radical **10**, generated by reductive cleavage of S-adenosyl methionine (SAM), abstracts the 3' hydrogen atom from GTP to give **11**, which then undergoes cyclization to give **13**. Reduction of this radical to **14**, by the purine liganded iron sulfur cluster followed by amination hydrolysis gives **15**. An α-ketol rearrangement to **16** completes the insertion of the purine C8 carbon into the C2'—C3' bond of the ribose. Ring opening of **16** followed by tautomerization gives **18**. Conjugate addition gives **19**. Loss of water, followed by tautomerization and ring closure completes the formation of the reaction product **6**.¹¹ While ribose and deoxyribose radicals have been extensively studied (DNA damage by radiation or radical-generating antibiotics, ribonucleotide reductase), addition of a C3' ribose centered radical to C8 of a purine has never been reported.¹³⁻¹⁵ The successful trapping of intermediate **14** would provide a critical test for the proposed mechanism.

Trapping of intermediate 14

The hydroxyl groups at the 2' and 3' positions of GTP are proposed to play an important role in the conversion of GTP to pterin (**15** to **16** and **19** to **20** in Figure 4). With the goal of trapping an early intermediate, 2',3'-dideoxyGTP **22** was tested as a substrate analog. The product observed in the MoaA/2',3'-dideoxyGTP reaction was 2 Da less than that of the substrate and had a UV-Vis spectrum similar to guanosine. Two mechanistic proposals were considered for the formation of this product (**24** or **28** in Figure 5).

HPLC comparison of the dephosphorylated enzymatic product with a reference sample of **25** eliminated **24** as the reaction product.

If **27** is the product structure, oxidation and dephosphorylation, followed by N-glycosyl bond cleavage would give **30** which could then be correlated with a reference sample. In the event, the dephosphorylated and acid hydrolyzed enzymatic product was found to be identical, by LC-MS, to a synthesized sample of **30** demonstrating that compound **27** is the product of the MoaA-2',3'-dideoxyGTP reaction.¹⁶ This result was independently supported by an elegant experiment involving the direct isolation and characterization of **14**.¹⁷

Trapping of the Intermediate 11

2'-chloroGTP **31** was explored as a trap for radical **11** (Figure 4). This trapping strategy was previously developed to study radical intermediates formed by ribonucleotide reductase, DNA irradiation, and radical-generating antibiotics.¹³⁻¹⁵

Analysis of the MoaA/2'-chloroGTP reaction demonstrated the formation of guanine and the absence of pterin **6**. A mechanistic analysis of this reaction is shown in Figure 6. Formation of the ribose radical **32** followed by β bond scission gives **33**. Glycosidic bond cleavage of **33** gives guanine **35** and suggests the formation of **34** which then eliminates triphosphate to

form **36**.¹⁸ The proposed ribose-derived product **36** was trapped by treating the reaction mixture with 3'-mercaptobenzoic acid and the resulting product was demonstrated to be identical to an authentic sample of **37** by LC-MS.¹¹

Mechanistic studies on the conversion of **15** to **6**

The carbon insertion into the ribose (**15** to **16** in Figure 4) could occur by a concerted α -ketol rearrangement or by a retroaldol/aldol sequence as shown in Figure 7. For the latter pathway, removal of the C2' hydroxyl group should allow us to trap intermediate **15**. For the concerted insertion, removal of the C2' hydroxyl group should result in the trapping of a late intermediate in the conversion of **15** to **6**.

The MoaA/2'-deoxyGTP reaction mixture was treated with phosphatase and analyzed by HPLC to reveal the formation of a fluorescent product with a UV-Vis spectrum similar to that of a pterin.^{19,20} Oxidation with KI/I₂ was not necessary to stabilize this product, which was identified as pterin **40** by comparison with a reference sample. (Figure 8A).¹¹ This is consistent with a concerted mechanism for the insertion reaction.

A mechanistic proposal for the formation of **40** is shown in Figure 8B and is consistent with the proposed conversion of **15** to **6** shown in Figure 4.

Conclusions

MoaA catalyzes the first step in molybdopterin biosynthesis in which GTP is converted to the pterin **6**.¹¹ This catalysis involves a remarkable rearrangement reaction in which the C8 of guanosine-5'-triphosphate (GTP) is inserted between the C2' and C3' carbon atoms of the GTP ribose. A mechanistic proposal for this remarkable transformation, unprecedented in ribose chemistry, is outlined in Figure 4. This proposal is now supported by the identification of the C3' of ribose as the site of initial radical formation, and by the successful trapping of **11**, **14**, and **19** using substrate analogs.

Acknowledgments

This research was supported by the Robert A. Welch Foundation (A-0034 to TPB).

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Highlights

- MoaA catalyzes the first step in molybdopterin biosynthesis
- Novel insertion of the purine C8 carbon between C2' and C3' of the GTP ribose
- Reaction triggered by radical formation at C3' of GTP
- Mechanistic proposal supported by intermediate trapping

Figure 1.

(A) Outline of the molybdopterin biosynthetic pathway. (B) ^{13}C - labeling studies show the remarkable carbon-insertion reaction catalyzed by MoaA.

Figure 2.
The active site of MoaA showing the two [4Fe-4S] clusters, GTP and 5'-deoxyadenosine (5'dA).

Figure 3.

The MoaA-catalyzed conversion of GTP **1** to PTP **6**. For ease of HPLC purification, **6** was treated with phosphatase and oxidized with KI/I₂. The oxidized compound **7** was characterized by NMR and LCMS analysis. **7** was also trapped by using o-(pentafluorobenzyl) hydroxylamine (PFBHA) to generate the corresponding oxime **8**.

Figure 4.

Mechanistic proposal for the MoxA-catalyzed reaction.

Figure 5.

Mechanistic analysis to suggest possible products from the reaction between MoaA and 2',3'-dideoxyGTP 22.

Figure 6.

Mechanistic analysis of the reaction between MoaA and 2'-chloroGTP **31**. Loss of chloride from **32** followed by reduction is also possible.

Figure 7. Mechanistic analysis of the C8 insertion reaction. Compound **16** could be formed by a concerted rearrangement of **15** or by a retroaldol/aldol sequence. Removal of the C2' hydroxyl would block the latter pathway.

Figure 8.

(A) The reaction between MoaA and 2'-deoxyGTP reaction. (B) Mechanistic proposal for the formation of the pterin **40**.