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Mechanistic Investigation of cPMP Synthase in Molybdenum Cofactor Biosynthesis Using an Uncleavable Substrate Analogue

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Abstract

Molybdenum cofactor (Moco) is essential for all kingdoms of life, plays central roles in various biological processes, and must be biosynthesized *de novo*. During its biosynthesis, the characteristic pyranopterin ring is constructed by a complex rearrangement of guanosine 5'-triphosphate (GTP) into cyclic pyranopterin monophosphate (cPMP) through the action of two enzymes, MoaA and MoaC. Recent studies revealed that MoaC catalyzes the majority of the transformation and produces cPMP from a unique cyclic nucleotide, 3',8-cyclo-7,8-dihydro-GTP (3',8-cH₂GTP). However, the mechanism by which MoaC catalyzes this complex rearrangement is largely unexplored. Here, we report the mechanistic characterization of MoaC using an uncleavable substrate analogue, 3',8-cH₂GMP[CH₂]PP, as a probe to investigate the timing of cyclic phosphate formation. Using partially active MoaC variants, 3',8-cH₂GMP[CH₂]PP was found to be accepted by MoaC as a substrate and was converted to an analogue of the previously described MoaC reaction intermediate, suggesting that the early stage of catalysis proceeds without cyclic phosphate formation. In contrast, when it was incubated with wt-MoaC, 3',8-cH₂GMP[CH₂]PP caused mechanism-based inhibition. Detailed characterization of the inhibited MoaC suggested that 3',8-cH₂GMP[CH₂]PP is mainly converted to a molecule (compound Y) with an acid-labile triaminopyrimidinone base without an established pyranopterin structure. MS analysis of MoaC treated with 3',8-cH₂GMP[CH₂]PP provided strong evidence that compound Y forms a tight complex with MoaC likely through a covalent linkage. These observations are consistent with a mechanism in which cyclic phosphate ring formation proceeds in concert with the pterin ring formation. This mechanism would provide a thermodynamic driving force to complete the formation of the unique tetracyclic structure of cPMP.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00857. Additional data and details of the characterization of 3',8-cH₂GMP[CH₂]PP, [α,β -methyleno]intermediate X, and 6-carboxypterin(PDF)

Molybdenum cofactor (Moco) is a ubiquitous enzyme cofactor that mediates redox reactions in the active sites of a number of enzymes. Moco-dependent enzymes play central roles in purine and sulfur catabolism and drug metabolism in mammals (including humans), anaerobic respiration in pathogenic bacteria, and nitrate assimilation in plants.^{1,2} Importantly, Moco must be synthesized *de novo* in cells, as it is chemically unstable and cannot be taken up as a nutrient.^{1,2} Due to this essential role, Moco biosynthesis has been linked to a number of human health problems. For example, genetic mutations in human Moco biosynthetic genes cause a fatal metabolic disorder, Moco deficiency.^{1,3} Recently, Moco biosynthesis in pathogenic bacteria has been gaining significant interest because Moco is critical for the virulence of bacterial pathogens such as *Mycobacterium tuberculosis* and *Pseudomonas aureus*.^{4–6} In fact, the pharmacological inhibition of Moco biosynthesis was shown to be effective for the treatment of latent tuberculosis in a mouse model.⁷ Therefore, it is important to understand the molecular details of Moco biosynthesis in bacteria and humans.

During Moco biosynthesis in *Escherichia coli*, the characteristic pyranopterin ring is constructed by a complex rearrangement of GTP into cPMP.⁸ This unusual transformation involves the insertion of the guanine C-8 between C-2' and C-3' of ribose (Figure 1A)⁹ and requires two enzymes, MoaA and MoaC.^{9–11} Conventionally, MoaA, a member of the radical *S*-adenosyl-L-methionine (SAM) superfamily,¹² was thought to be responsible for the formation of the pyranopterin backbone structure.^{11,13–17} However, recent characterization of the MoaA product by NMR and X-ray crystallography and functional characterization of MoaC revealed that MoaA catalyzes the transformation of GTP to 3',8-cH₂GTP (**2**; Figure 1A) and that the remaining conversion to cPMP is catalyzed by MoaC.^{18,19} Because of this unexpected finding regarding the catalytic function of MoaC, the mechanism of the MoaC-catalyzed transformation of 3',8-cH₂GTP to cPMP has been largely unexplored.

Conventionally, MoaC was thought to be responsible only for the formation of the cyclic phosphate moiety of cPMP on the basis of the proposal that the MoaC substrate is pyranopterin triphosphate (**5**; Figure 1B).^{14,16} However, in the revised function (Figure 1A), the MoaC substrate is now known to be 3',8-cH₂GTP and the catalysis involves not only cyclic phosphate formation but also a complex rearrangement of the ribose and base moieties of 3',8-cH₂GTP.^{18,19} This significant revision in the role of MoaC has raised a question about the timing of cyclic phosphate formation in the complex function of MoaC catalysis. The timing of cyclic phosphate formation is of mechanistic interest because it involves the cleavage of a high-energy phosphodiester bond and could provide a thermodynamic driving force for the reaction. In our most recent working hypothesis (Figure 1C),¹⁹ based mainly on the X-ray crystal structures of MoaC in complex with 3',8-cH₂GTP and cPMP, we proposed that cyclic phosphate formation is one of the last steps. In this

model, MoaC catalysis is initiated by either amination hydrolysis or C2'–C3' bond cleavage to yield an as-yet structurally uncharacterized intermediate X followed by a rearrangement to yield N-hexosyl triaminopyrimidinone (**6**), which is then converted to cPMP by formation of the pterin and cyclic phosphate rings. While the mechanism is consistent with the X-ray crystal structures of MoaC, there is currently no enzymological evidence for the timing of the cyclic phosphate ring formation. As such, we set out to probe the mechanism of this transformation. Here, we report the results of our investigation using uncleavable substrate analogue 3',8-cyclo-7,8-dihydro-guanosine [(α,β)-methyleno]triphosphate (3',8- $\text{cH}_2\text{GMP}[\text{CH}_2]\text{PP}$, **7**; Figure 1D). Our characterization revealed that the reaction yielded a species with an acid-labile triaminopyrimidinone base with no pterin structure that is either sufficiently electrophilic to form covalent modification of MoaC or spontaneously converted to a pterin molecule in the absence of MoaC. These characteristics of the observed reaction product are consistent with the uncleavable analogue of N-hexosyl triaminopyrimidinone (**6**). These observations also suggest that in MoaC catalysis the cyclic phosphate and pterin rings are formed in a concerted manner, as opposed to the previous notion, where cyclic phosphate formation takes place independent of pterin ring formation.^{14,16} These studies provide the first enzymological insights into the late stages of MoaC catalysis.

EXPERIMENTAL PROCEDURES

General Methods

Guanosine 5'-triphosphate (GTP), S-adenosyl-L-methionine (SAM), dithiothreitol (DTT), and sodium dithionite were purchased from Sigma-Aldrich. Guanosine 5'-[(α,β)-methyleno]triphosphate (GMP[CH₂]PP) was purchased from Jena Biosciences. Nonlinear least-squares fitting of kinetic data was carried out using KaleidaGraph software (Synergy Software, Reading, PA). N-Terminally His₆-tagged *Staphylococcus aureus* MoaA and *E. coli* MoaC were expressed and purified from *E. coli* as described previously.^{18,19} Anaerobic experiments were carried out in an UNILab workstation glovebox (MBAun, Stratham, NH) maintained at 10 ± 2 °C with an O₂ concentration < 0.1 ppm. 3',8- cH_2GTP was prepared as described previously.¹⁸ All MoaA and MoaC assays and HPLC sample preparations were performed at 25 °C under strict anaerobic conditions (O₂ < 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.

Preparation of 3',8- $\text{cH}_2\text{GMP}[\text{CH}_2]\text{PP}$

To prepare 3',8- $\text{cH}_2\text{GMP}[\text{CH}_2]\text{PP}$, MoaA (65 μM) was incubated with SAM (1 mM), sodium dithionite (2 mM), and GMP[CH₂]PP (0.3 mM) in assay buffer (50 mM Tris-HCl, pH 7.6, 2 mM MgCl₂, 5 mM DTT, and 0.3 M NaCl) at 25 °C. After incubation for 120 min, MoaA was removed by filtration with a 10 kDa MWCO filter (Millipore). This reaction typically yields a solution with 80 μM 3',8- $\text{cH}_2\text{GMP}[\text{CH}_2]\text{PP}$, which was used for subsequent MoaC assays without further purification. The amount of 3',8- $\text{cH}_2\text{GMP}[\text{CH}_2]\text{PP}$ was quantified based on its derivatization to dimethylpterin as described previously.¹⁸

Anaerobic HPLC Characterization of Products of the MoaC Assay with 3',8-cH₂GMP[CH₂]PP as Substrate

MoaC assays were performed in the presence of 25 or 160 μ M wt-MoaC or 160 μ M MoaC variants and 52 μ M 3',8-cH₂GMP[CH₂]PP. In these assays, 3',8-cH₂GMP[CH₂]PP was prepared as described above without further purification. The presence of residual SAM and GMP[CH₂]PP did not affect the MoaC assay. After 120 min of incubation, the reactions were quenched by addition of a stoichiometric volume of anaerobic MeOH, and the protein precipitant was removed after pelleting by centrifugation at 13 000 rpm for 5 min. The supernatant was analyzed by ion-pairing HPLC under anaerobic conditions using anaerobically prepared solvents and buffers under a flow of Ar gas. An Alltech Apollo C18 column (4.6 $\times$ 250 mm, 2.7 μ m, part no. 36511) was initially equilibrated in 0.1 M KH₂PO₄, pH 6.0, 8 mM tetrabutylammonium hydrogen sulfate (Solvent A), to which 60 μ L of the assay supernatant was applied. The elution was performed at a flow rate of 1.0 mL/min using solvents A and B (0.1 M KH₂PO₄, pH 6.0, 8 mM tetrabutylammonium hydrogen sulfate, 30% (v/v) ACN): 0% B for 6.5 min, 0–20% B for 6.5 min, 20–40% B for 13 min, and 40–100% B for 8 min. Chromatography was monitored by the L-2455 diode array detector.

IC₅₀ Determination of MoaC Inhibition by 3',8-cH₂GMP[CH₂]PP

wt-MoaC (0.2 μ M) was incubated with 3',8-cH₂GTP (15 μ M) and specified concentrations of 3',8-cH₂GMP[CH₂]PP in assay buffer at 25 °C. The reaction was then quenched at 2, 4, 6, 8, and 10 min with 0.1 volume of 25% TCA. cPMP was quantified by HPLC after its conversion to compound Z.¹⁸

Characterization of 3',8-cH₂GMP[CH₂]PP-Treated MoaC

wt-MoaC (10 μ M) was incubated for 30 min at 25 °C in the presence or absence of 3',8-cH₂GMP[CH₂]PP (19.3 μ M). Subsequently, both samples were buffer-exchanged over a 10 kDa MWCO filter. This buffer exchange resulted in a 625-fold dilution of the small molecules in the protein fraction. MoaC (17 kDa) was found to be exclusively in the protein fraction based on SDS-PAGE. To test the activity, the resulting MoaC was diluted to 0.1 μ M and incubated with 3',8-cH₂GTP (10.6 μ M) in assay buffer at 25 °C. The reaction was quenched at 2, 4, 6, 8, and 10 min with 0.1 volume of 25% TCA, and cPMP was quantified by HPLC after its conversion to compound Z.¹⁸

To investigate for the presence of acid-labile triaminopyrimidinone, the retentate or filtrate after ultrafiltration of the solution from the MoaC assay with 3',8-cH₂GMP[CH₂]PP was acidified with 0.1 volume of anaerobic 0.5 M HCl to quench the reaction. The resulting mixture was incubated at 95 °C for 5 min to facilitate hydrolysis, followed by an addition of 6 μ L of 1 M NaOH to adjust the pH to 8.5. The resulting solution was combined with 25 μ L 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 μ L) of the supernatant was injected into the HPLC. The chromatography was performed by an isocratic elution with 92.5% 20 mM sodium acetate, pH 6.0, 7.5% MeOH at 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 dimethylpterin was eluted at 5.6 min. For quantitation, a standard

curve was generated using an authentic sample of trimethylpyrimidinone (Sigma) derivatized under the identical conditions.

To investigate for the presence of pterin compounds, the retentate or filtrate after the ultrafiltration of the solution from the MoaC assay with 3',8-cH₂GMP[CH₂]PP was mixed with 0.1 volume of an anaerobic solution of I₂ (1% w/v), KI (2% w/v), and TCA (25% w/v) and incubated for 60 min at 22 °C. The resulting mixture was analyzed by ion-pairing HPLC using the conditions described above except that elution was performed by a linear gradient of 0–60% B over 20 min at 1.2 mL/min. Chromatography was monitored by an diode array detector and a fluorescence detector (ex. 365 nm, em. 445 nm). For LC-MS analysis, the HPLC peak at 4.7 min from the MoaC reaction with 3',8-cH₂GMP[CH₂]PP was collected and batch purified with SP-Sephadex (ammonium form) to remove tetrabutylammonium ions. The resulting sample was lyophilized, resuspended in 20 mM ammonium formate, pH 8.0, and analyzed in negative ion mode using an Agilent 6224 LC-ESI/TOF-MS equipped with an Agilent Poroshell 120 C18 column (2.1 × 75 mm, 2.7 μm, part no. 697775-906). The elution was performed at a flow rate of 0.15 mL/min using 20 mM ammonium formate, pH 8.0, (solvent A) and MeOH (solvent B): 0–2% B for 10 min, 2–50% B for 7 min, and 50–100% B for 5 min. Data was processed using Agilent MassHunter software. The amount of 6-carboxypterin was quantified using an authentic standard (Sigma) and the reported molar extinction coefficient, $\epsilon_{344\text{nm}} = 7410 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 5.0.⁹

ESI-MS Analysis of MoaC

wt-MoaC (5 μM) was incubated for 120 min at 25 °C in the presence or absence of 3',8-cH₂GMPcPP (10 μM). Subsequently, samples were buffer-exchanged in 150 mM ammonium bicarbonate, pH 8.0, over a 10 kDa MWCO filter. This buffer exchange resulted in a 4096-fold dilution of the small molecules in the protein fraction. Then, the samples were diluted to 10 μM in 20% acetonitrile/30 mM ammonium bicarbonate, pH 8.0, and directly infused through a fused-silica capillary at 4 μL/min into a Waters Synapt G2 QTOF mass spectrometer operating in positive ionization mode through an electrospray interface. Source temperature was set to 80 °C, and the N₂ nebulizing gas was set to 4 L/min. Approximately 30 full MS scans from *m/z* 50 to 2000 were collected for each condition. Spectra were summed and then deconvoluted with the Waters MaxEnt1 algorithm. Horse myoglobin was infused immediately prior to the test samples, and a static mass correction (–1.5 Da) was applied to all test samples based on the net difference from the expected myoglobin mass.

RESULTS AND DISCUSSION

3',8-cH₂GMP[CH₂]PP has a methylene bridge in place of an oxygen between the α and β phosphate groups and therefore is expected to block cyclic phosphate formation. On the basis of our current working hypothesis (Figure 1C),¹⁹ the early steps of MoaC catalysis involve the rearrangement of the base and ribose moieties and one of the last steps is cyclic phosphate ring formation. Therefore, when it is incubated with MoaC, 3',8-cH₂GMP[CH₂]PP is expected to be converted to an advanced reaction intermediate immediately prior to cyclic phosphate formation. Characterization of the trapped intermediate will provide insight into the timing of cyclic phosphate formation. To this end,

we first prepared 3',8-cH₂GMP[CH₂]PP from guanosine [(α,β)-methylene]triphosphate (GMP[CH₂]PP) using MoaA. Since the triphosphate moiety is not involved in MoaA catalysis, MoaA was expected to accept GMP[CH₂]PP and convert it into 3',8-cH₂GMP[CH₂]PP. Thus, GMP[CH₂]-PP was incubated with MoaA under strict anaerobic conditions (O₂ < 0.1 ppm), and the reaction products were analyzed anaerobically by ion-pairing HPLC. These analyses revealed a peak that migrated faster than 3',8-cH₂GTP (Figure 2A, compare traces 1 and 9) and exhibited a characteristic UV-vis absorption spectrum essentially identical to that of 3',8-cH₂GTP¹⁸ (Figure 2B). LC-MS analysis of this product was also consistent with the formation of 3',8-cH₂GMP[CH₂]PP (Figure S1).

The resulting 3',8-cH₂GMP[CH₂]PP was then used to investigate its reaction with MoaC. Initially, the reaction of wt-MoaC with 3',8-cH₂GMP[CH₂]PP was analyzed for the potential products by HPLC under anaerobic conditions. However, in such assays, no products were detectable (Figure 2A, trace 6), even though depletion of 3',8-cH₂GMP[CH₂]PP was observed. The extent of 3',8-cH₂GMP[CH₂]PP depletion was dependent on the ratio between the concentrations of 3',8-cH₂GMP[CH₂]PP and wt-MoaC; complete depletion was observed when the amount of wt-MoaC (160 μ M) was in excess of 3',8-cH₂GMP[CH₂]PP (52 μ M), whereas the depletion was minimal when wt-MoaC (25 μ M) was substoichiometric to 3',8-cH₂GMP[CH₂]PP (52 μ M). These observations suggested that the product of the reaction between MoaC and 3',8-cH₂GMP[CH₂]PP is tightly bound to MoaC and not readily released from the active site.

To obtain further insight into the reactivity of 3',8-cH₂GMP[CH₂]PP with MoaC, we next tested whether 3',8-cH₂GMP[CH₂]PP is converted to an analogue of a previously reported MoaC reaction intermediate. The conversion of 3',8-cH₂GTP to cPMP by wt-MoaC proceeds via intermediate X (Figure 1B). Because the formation of intermediate X precedes cyclic phosphate formation,¹⁹ we tested if 3',8-cH₂GMP[CH₂]-PP is converted to an (α,β)-methylene analogue of intermediate X. To this end, we used active-site variants of MoaC (K51A- and K131A-MoaC),¹⁹ which catalyze the transformation of 3',8-cH₂GTP to intermediate X but are incapable of converting intermediate X to the final product, cPMP. When 3',8-cH₂GMP[CH₂]PP was incubated with K51A-MoaC or K131A-MoaC, 3',8-cH₂GMP[CH₂]PP was quantitatively converted into a molecule that eluted earlier than 3',8-cH₂GMP[CH₂]PP (Figure 2A, traces 2 and 5) and exhibited an absorption spectrum essentially identical to that of intermediate X (Figure 2B). Characterization of this compound by chemical derivatization was also consistent with the formation of [(α,β)-methylene] intermediate X (Figure S2). No reaction was observed for the other variants, H77A- and D128A-MoaC (Figure 2A), which are incapable of forming intermediate X with the natural substrate, 3',8-cH₂GTP.¹⁹ These observations suggest that 3',8-cH₂GMP[CH₂]PP serves as a substrate of MoaC and undergoes early steps of MoaC catalysis at least up until intermediate X.

The ability of 3',8-cH₂GMP[CH₂]PP to serve as a MoaC substrate is also consistent with the X-ray crystal structure of MoaC.¹⁹ In the MoaC structure in complex with 3',8-cH₂GTP, the triphosphate moiety of 3',8-cH₂GTP is exposed to solvent and forms only one H-bond interaction between α -phosphate and H77. Therefore, it is reasonable for 3',8-

cH₂GMP[CH₂]PP to bind the MoaC active site in a manner identical to that of 3',8-cH₂GTP and undergo the early steps of the MoaC reaction that modify the base and sugar moieties.

On the basis of the above observation, the most reasonable interpretation of the results from the wt-MoaC reaction with 3',8-cH₂GMP[CH₂]PP is that 3',8-cH₂GMP[CH₂]PP is converted to an analogue of an advanced intermediate that binds to MoaC tightly without release. In this case, 3',8-cH₂GMP[CH₂]PP should serve as a mechanism-based inhibitor of wt-MoaC. Thus, the inhibitory effects of 3',8-cH₂GMP[CH₂]PP were investigated by performing MoaC assays using its natural substrate, 3',8-cH₂GTP, in the presence of varying concentrations of 3',8-cH₂GMP[CH₂]PP. As a result, dose-dependent inhibition of MoaC activity was observed with an IC₅₀ value of $8.6 \pm 2.3 \mu\text{M}$ (Figure 3A). To test the reversibility of the inhibition, MoaC ($10 \mu\text{M}$) was preincubated with 3',8-cH₂GMP[CH₂]PP ($19.3 \mu\text{M}$), followed by removal of small molecules by repeating the ultrafiltration. The resulting MoaC did not contain 3',8-cH₂GMP[CH₂]PP above the detection limit ($0.03 \mu\text{M}$) and was still potently inhibited to an extent comparable to that prior to the removal of 3',8-cH₂GMP[CH₂]PP (Figure 3B). These observations are consistent with the conclusion that 3',8-cH₂GMP[CH₂]PP causes MoaC inhibition by a tight-binding or covalent mechanism.

To obtain further insight into the chemical species causing the inhibition, the product of the MoaC reaction with 3',8-cH₂GMP[CH₂]PP was characterized. Although we initially attempted to release the product from MoaC under various conditions, such as protein denaturing in acid or ethanol, no small molecule products were observed by anaerobic HPLC analysis. Attempts to detect the product using carbonyl-reactive reagents, such as *O*-(4-nitrobenzyl)hydroxylamine and *O*-(pentafluorobenzyl)hydroxylamine, did not result in detectable levels of small molecule derivatives. Therefore, we employed two chemical methods to detect characteristic structures expected in MoaC reaction intermediates. In the first method, pyranopterins, such as compound **5**, are detected by oxidative conversion to pterins using KI/I₂.^{2,8} The second method was to detect acid-labile triaminopyrimidinone base, which is expected to be present in precursors such as compound **6** (Figure 1C).^{18,20} In these analyses, 3',8-cH₂GMP[CH₂]PP-treated wt-MoaC was first separated from small molecules using ultrafiltration (10 kDa MWCO). Then, the filtration retentate, the fraction containing macromolecules >10 kDa in size, was analyzed for the presence of pyranopterin or acid-labile triaminopyrimidinone by their conversion to fluorescence derivatives, pterin and dimethylpterin, respectively.¹⁸ The resulting derivatives were quantified by HPLC. In this analysis, dimethylpterin was observed in an amount stoichiometric to MoaC (1.2 ± 0.03 equiv per MoaC; Figure 4), whereas no significant amount of pterin compounds was detected. These results indicated that the inhibitory species contains an acid-labile triaminopyrimidinone but not a pyranopterin ring and that it bound MoaC tightly.

The tight binding of the acid-labile triaminopyrimidinone-containing product (compound Y) was also demonstrated by MS analysis (Figure 5). In this experiment, MoaC was incubated with 3',8-cH₂GMP[CH₂]PP as described above, thoroughly buffer exchanged using ultrafiltration into ammonium bicarbonate buffer (pH 8.0), and subjected to molecular weight determination by ESI-MS in 20% acetonitrile. The resulting MoaC retained 0.6 ± 0.1 equiv of compound Y based on the chemical derivatization assay. This sample was then subjected to ESI-QTOF MS analysis by direct infusion. The deconvoluted mass spectra

revealed two clusters of mass signals (Figure 5). One corresponded to MoaC and its sodium and ammonium salts. The other corresponded to a species with a molecular weight 504 ± 1 Da greater than that of MoaC, which was absent in a control without 3',8-cH₂GMP[CH₂]PP. The ratio between the intensities of the MS signals for unmodified vs modified MoaC (~1:2) was consistent with the stoichiometry of compound Y to MoaC (0.6 ± 0.1 equiv). Therefore, these observations suggest that the reaction of MoaC with 3',8-cH₂GMP[CH₂]PP yields compound Y, which tightly binds MoaC, likely through a covalent bond.

The ultrafiltration filtrate of the reaction, the fraction containing small molecules (<10 kDa), was also analyzed for the acid-labile triaminopyrimidinone and pterins. This analysis revealed the presence of only a small amount of acid-labile triaminopyrimidinone (<10% of MoaC; Figure 4), consistent with the tight binding of compound Y to MoaC. As for the pterin product, in contrast to the protein fraction, the small molecule fraction contained a detectable but low level of pterin species (Figure 4). This pterin species was absent in negative controls without MoaC or 3',8-cH₂GMP[CH₂]PP (Figure 6A, traces 2 and 3), suggesting that it is derived from the product of the reaction of MoaC with 3',8-cH₂GMP[CH₂]PP. Upon further UV-vis and ESI-MS analyses of this species and comparison with an authentic standard (Figures 6B and S3), the observed compound was identified as 6-carboxypterin (**8**; Figure 6C). However, quantitation of this species using an authentic standard revealed that the amount of 6-carboxypterin formed was small: $8.7 \pm 3.2\%$ of MoaC. This is significantly lower than the amount of compound Y bound to MoaC: 60–100% relative to MoaC. Therefore, together with the fact that 6-carboxypterin was the only pterin product detected, this observation suggests that in the reaction of wt-MoaC with 3',8-cH₂GMP[CH₂]PP the pterin ring formation is significantly perturbed.

On the basis of all of these observations, we propose the reaction model shown in Figure 7A, in which the product of the reaction of MoaC with 3',8-cH₂GMP[CH₂]PP is [(α,β)-methyleno]N-hexosyl-triaminopyrimidinone (**9**; Figure 7A). The resulting **9** stalls in the active site, the majority of which results in the formation of a covalent complex with MoaC through a nucleophilic attack of an active site amino acid residue to the electrophilic α,β -unsaturated ketone in **9** (Figure 7B). This MoaC–**9** conjugate is then dehydrated to give an adduct with a molecular weight of 503 Da, which is consistent with the observed mass increase of 504 ± 1 Da.

The reaction yielded a small amount of pterin compound (6-carboxypterin; see Figure S4 for a possible mechanism of formation). However, considering that this amount was significantly lower than that of compound Y (9% vs >60%), the observation suggests that pterin ring formation is significantly perturbed. Therefore, these combined observations are most consistent with the concerted formation of the pterin and cyclic phosphate rings (Figure 7C). In this mechanism, pterin ring formation is coupled to the thermodynamically favored and likely irreversible cyclic phosphate formation. Therefore, this mechanism allows MoaC to use phosphodiester bond cleavage as a thermodynamic driving force to catalyze pterin ring formation and guide the complex rearrangement reaction to completion. Importantly, this mechanism is distinct from a previously accepted mechanism that was widely believed prior to the revision of MoaC's function, in which pterin ring formation takes place independently of and prior to cyclic phosphate formation.^{14–16} Therefore, the

current observations also highlight the importance of the mechanistic coupling of the formation of the pterin and cyclic phosphate rings.

In conclusion, the data presented here provide the first insights into the late stages of the MoaC-catalyzed conversion of 3',8-cH₂GMP[CH₂]PP to cPMP. Using 3',8-cH₂GMP-[CH₂]PP as the mechanistic probe, our observations suggest that cyclic phosphate formation is the last step of catalysis coupled to pterin ring formation and that it likely provides the thermodynamic driving force for the formation of the characteristic tetracyclic structure of cPMP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Moco	molybdenum cofactor
GTP	guanosine 5'-triphosphate
3',8-cH₂GTP	3',8-cyclo-7,8-dihydro-GTP
cPMP	cyclic pyranopterin monophosphate
GMP[CH₂]PP	guanosine [(α,β)-methylene]triphosphate
HPLC	high-pressure liquid chromatography
TCA	trichloroacetic acid

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Figure 1.

Moco biosynthesis and the formation of cPMP. (A) Moco biosynthetic pathway. The symbols on GTP and cPMP indicate the origin of the carbon and nitrogen atoms in cPMP, as determined by isotope labeling studies.⁹ cPMP may also be in a hydrate form in solution.²¹ (B) Structure of the previously proposed MoaC substrate, pyranopterin triphosphate. (C) Current working hypothesis for the MoaC-catalyzed conversion of 3',8-cH₂GTP to cPMP.¹⁹ The proposed structural candidates for intermediate X are shown. (D) Uncleaveable analogue of 3',8-cH₂GTP (3',8-cH₂GMP[CH₂]PP).

Figure 2.

Characterization of the products from assays of wt and variants of MoaC with 3',8-cH₂GMP[CH₂]PP. (A) Anaerobic ion-pairing HPLC analysis. wt-MoaC (160 μ M) or a MoaC variant (160 μ M) was incubated with 3',8-cH₂GMP[CH₂]PP (52 μ M) and analyzed by HPLC with tetrabutylammonium hydrogen sulfate as an ion-pairing agent under a Ar atmosphere (traces 2–6). Chromatographs for 3',8-cH₂GMP[CH₂]PP, cPMP, intermediate X, and 3',8-cH₂GTP under the same conditions are shown for comparison (traces 1 and 7–9, respectively). (B) UV–vis absorption spectra of 3',8-cH₂GMP[CH₂]PP (solid black line) and [(α,β)-methylene]-intermediate X (dotted black line) determined from diode array detection of HPLC traces 1 and 2 in (A), respectively. Also shown are the spectra for 3',8-cH₂GTP (solid gray line) and intermediate X (dashed gray line) for comparison.

Figure 3.

Characterization of wt-MoaC reaction with 3',8-cH₂GMP-[CH₂]PP. (A) Dose-dependent inhibition of MoaC by 3',8-cH₂GMP-[CH₂]PP. MoaC activity was determined by incubating MoaC (0.2 μM) with 3',8-cH₂GTP (15 μM) in the presence of varying concentrations of 3',8-cH₂GMP-[CH₂]PP. The solid line is a fit to the equation²² $v_i/v_o = 1/((1 + 10^{\log(I) - \log(IC_{50})}) \times 1.6)$ with an IC₅₀ value of 8.6 ± 2.3 μM. The activity of MoaC preincubated with GMP-[CH₂]PP, which should not have significant inhibitory effects on MoaC activity, is shown as a control. (B) Activity of 3',8-cH₂GMP-[CH₂]PP-treated MoaC after removal of unreacted 3',8-cH₂GMP-[CH₂]PP. MoaC (10 μM) was incubated with 3',8-cH₂GMP-[CH₂]PP (19.3 μM) or assay buffer, followed by the removal of unreacted 3',8-cH₂GMP-[CH₂]PP using a 10 kDa MWCO filter. The activity of the resulting MoaC was determined based on its ability to catalyze the conversion of 3',8-cH₂GTP to cPMP.

Figure 4. Quantitation of triaminopyrimidinone- and pterin-containing species in the wt-MoaC assay with 3',8-cH₂GMP[CH₂]PP. Chemical analysis of the wt-MoaC reaction with 3',8-cH₂GMP[CH₂]PP after separation of protein-bound and unbound molecules. MoaC assay with 3',8-cH₂GMP[CH₂]PP was performed as in Figure 3B, and proteins >10 kDa were separated from small molecules by ultra-filtration. Each fraction was treated with TCA and butane-2,3-dione for detection of molecules with acid-labile triaminopyrimidinone (red bars) or with TCA and KI/I₂ to detect pterin molecules (green bars).

Figure 5.

MS analysis of wt-MoaC with and without treatment with 3',8-cH₂GMP[CH₂]PP. Deconvoluted MS spectra for wt-MoaC (A) and wt-MoaC treated with 3',8-cH₂GMP[CH₂]PP (B) are shown. The 3',8-cH₂GMP[CH₂]PP-treated sample was prepared as described in the caption to Figure 3 and buffer exchanged into 150 mM ammonium bicarbonate, pH 8.0, using ultrafiltration. The resulting sample was diluted into 30 mM ammonium bicarbonate, pH 8.0, 20% acetonitrile and directly injected to ESI-MS.

Figure 6.

Characterization of pterin species observed in the small molecule fraction (<10 kDa) of the wt-MoaC assay with 3',8-cH₂GMP[CH₂]PP. (A) Ion-pairing HPLC analysis (ex. 365 nm, em. 445 nm) of the small molecule fraction of the MoaC assay with 3',8-cH₂GMP[CH₂]PP after TCA/KI/I₂ treatment. A control without 3',8-cH₂GMP[CH₂]PP was performed using products of the MoaA reaction without GMP[CH₂]PP so that it contains all other components (e.g., SAM and dithionite; see Experimental Procedures for detail). Compound Z, the oxidized derivative of cPMP, in traces 1 and 2 is derived from GTP co-purified with MoaA.¹⁸ (B) UV-vis spectra of compound Z and the 6-carboxypterin authentic sample observed in the MoaC assay (the peak at 7.2 min in trace 1 of (A)). (C) Structure of 6-carboxypterin.

Figure 7.

Reaction of 3',8-cH₂GMP[CH₂]PP with wt-MoaC. (A) Proposed mechanism of the reaction of 3',8-cH₂GMP[CH₂]PP with wt-MoaC. 3',8-cH₂GMP[CH₂]PP is converted to compound Y with acid-labile triaminopyrimidinone base, such as [(α,β -methylene)*N*-hexosyl]triaminopyrimidinone (**9**). Accumulation of such a molecule in the active site could result in tight binding or covalent inhibition of MoaC (top path). The formation of a small amount of 6-carboxypterin is explained by the release of a minor amount of **9** to solution (bottom path; see Figure S4 for detail). (B) Possible mechanism of the covalent linking of **9** to MoaC. (C) Proposed mechanism of the MoaC-catalyzed formation of the tetracyclic structure of cPMP by a concerted cyclization of the pterin and cyclic phosphate rings.