Published in final edited form as:

J Am Chem Soc. 2020 May 20; 142(20): 9314–9326. doi:10.1021/jacs.0c01200.

Mechanism of rate acceleration of radical C-C bond formation reaction by a radical SAM GTP 3',8-cyclase

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Abstract

While the number of characterized radical *S*-adenosyl-L-methionine (SAM) enzymes is increasing, the roles of these enzymes in radical catalysis remain largely ambiguous. In radical SAM enzymes, the slow radical initiation step kinetically masks the subsequent steps, making it impossible to study the kinetics of radical chemistry. Due to this kinetic masking, it is unknown whether the subsequent radical reactions require rate acceleration by the enzyme active site. Here, we report the first evidence that a radical SAM enzyme MoaA accelerates the radical-mediated C-C bond formation. MoaA catalyzes an unprecedented 3',8-cyclization of GTP into 3',8-cyclo-7,8-dihydro-GTP (3',8-cH₂GTP) during the molybdenum cofactor (Moco) biosynthesis. Through a series of EPR and biochemical characterization, we found that MoaA catalyzes a shunt pathway in which an on-pathway intermediate, GTP C-3' radical, abstracts H-4' atom from (4' *R*)-5'-deoxyadenosine (5'-dA) to transiently generate 5'-deoxyadenos-4'-yl radical (5'-dAC4'•) that is subsequently reduced stereospecifically to yield (4' *S*)-5'-dA. Detailed kinetic characterization of the shunt and the main pathways provided the comprehensive view of MoaA kinetics and determined the rate of the on-pathway 3',8-cyclization step as $2.7 \pm 0.7 \text{ s}^{-1}$. Together with DFT calculations, this observation suggested that the 3',8-cyclization by MoaA is accelerated by 6 ~ 9 orders of magnitude. Further experimental and theoretical characterizations suggested that the rate acceleration is achieved mainly by constraining the triphosphate and guanine base positions while leaving the ribose flexible, and a transition state stabilization through H-bond or electrostatic interactions with the positively charged R17 residue. This is the first evidence for rate acceleration of radical reactions by a radical SAM enzyme and provides insights into the mechanism by which radical SAM enzymes accelerate radical chemistry.

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Supporting Information

Supplementary methods for expression and purification of MoaA and MoaC and their variants. Supplementary tables for EPR simulation and DFT calculations. Supplementary figures for EPR characterization at 15 K of MoaA assays in different buffers, power-saturation experiments, NMR characterization of (4' *R*)-5'-dA and (4' *S*)-5'-dA, LCMS of 5'-dA generated with [3'-D]GTP or in D₂O, MoaA assays with [4'-²H]SAM, fitting results with various kinetic models, characterization of R17K-MoaA assays, MS and NMR data for SAM isotopologs. The Supporting Information is available free of charge on the ACS Publications website.

Supplementary methods, tables and figures (PDF)

Graphical Abstract

Keywords

Radical SAM enzymes; molybdenum cofactor biosynthesis; MoaA; GTP 3', 8-cyclase

Introduction:

The scope of enzyme-catalyzed radical reactions is expanding especially since the emergence of radical *S*-adenosyl-L-methionine (SAM) enzymes¹. These enzymes catalyze unique and chemically challenging reactions by free-radical mediated mechanisms through reductive cleavage of SAM using oxygen sensitive [4Fe-4S] clusters (Figure 1A)²⁻³. Despite the increasing number of characterized radical SAM enzyme-catalyzed radical reactions, the role of enzyme active site environment in such radical catalysis remains ambiguous. In particular, it is unknown if radical chemistry requires rate acceleration by radical SAM enzymes. This significant knowledge gap is caused at least partly by the fact that all the radical chemistry is kinetically masked by the preceding slow radical initiation step, either the reductive SAM cleavage which was estimated to have ~ 390 mV redox potential gap⁴, or any physical steps such as protein conformational change to achieve a catalytically relevant SAM or substrate orientation. As a result, subsequent radical chemistry becomes mechanistically intractable. Here, using MoaA, an enzyme in Molybdenum cofactor (Moco) biosynthetic pathway, we report the first determination of the rate constant for the radical-mediated C-C bond formation in radical SAM enzymes. This work provides, to our knowledge, the first experimental evidence that a radical SAM enzyme accelerates a radical mediated reaction.

Moco is a redox enzyme cofactor found in all domains of life⁵⁻⁹. Moco-dependent enzymes play key roles in many biological pathways critical for life including purine and sulfur catabolism, anaerobic respiration, and nitrate assimilation⁶. While a salvage pathway was recently suggested in nematodes¹⁰, most organisms are incapable of taking up Moco as a nutrient due to the cofactor's chemical instability, making the *de novo* Moco biosynthesis an essential process. In humans, Moco biosynthesis is essential for healthy development of

brains. Inability to biosynthesize Moco causes fatal metabolic disorder, Moco deficiency (MoCD)¹¹. In bacteria, some of the Moco-dependent enzymes are involved in the virulence of several clinically important pathogens, such as *Mycobacterium tuberculosis*¹² and *Pseudomonas aeruginosa*¹³.

In all organisms, the characteristic pterin backbone structure of Moco is biosynthesized by a set of conserved enzymes through the transformation of GTP into cyclic pyranopterin monophosphate (cPMP) (Figure 1B)^{7, 14}. In bacteria, this transformation is catalyzed by two enzymes, MoaA and MoaC. MoaA catalyzes the transformation of GTP into an oxygen sensitive intermediate, 3',8-cyclo-7,8-dihydro-GTP (3',8- cH₂GTP)¹⁵, which is then transformed into cPMP by a complex rearrangement catalyzed by MoaC (Figure 1B)¹⁶.

The MoaA catalysis proceeds through a hydrogen atom abstraction from the 3'-position of GTP by 5'-dA• (Figure 1C)¹⁵. The resulting GTP C-3' radical (GTP-C3'•) attacks C-8 of guanine to generate a 3',8-cyclo-GTP aminyl radical, which is converted to 3',8-cH₂GTP by transfers of an electron and a proton. Among the steps after SAM cleavage, the addition of GTP-C3'• to C-8 is likely the most chemically challenging step. Nucleotide C-3' radicals have been reported as intermediates of several biochemical reactions, including the reactions catalyzed by ribonucleotide reductase (RNR), and an oxidative damage of DNA. In RNR, C-3' radical facilitates dissociation of 2'-OH during the formation of deoxynucleotides¹⁷⁻¹⁹. Perturbations in the active site that prevent the completion of RNR catalysis most frequently result in base dissociation²⁰⁻²². C-3' radical-mediated base dissociation was also reported for oxidative damage of DNA²³. The reactivity of nucleotide C-3' radical in oligonucleotides has been studied using photoactivatable nucleotides²⁴, in which C-3' radicals caused strand scission and base dissociation^{23, 25}. In any of these systems, 3',8-cyclization has never been observed. Thus, the addition of GTP-C3'• to C-8 is an unprecedented reactivity of C-3' radical in either biological or synthetic reactions. The mechanism by which MoaA catalyzes this unprecedented C3'-C8 cyclization is unknown.

In this work, we report comprehensive kinetic and spectroscopic characterization of the MoaA reaction, which revealed that the MoaA catalysis diverges to two pathways; the normal pathway yields 3',8-cH₂GTP and (4' *R*)-5'-dA and a shunt pathway yields (4' *S*)-5'-dA via 5'-deoxyadenos-4'-yl radical (5'-dA-C4'•). The presence of this shunt pathway allowed determination of the rate constant for the on-pathway 3',8-cyclization step to be $2.7 \pm 0.7 \text{ s}^{-1}$. Together with DFT calculation, these observations suggested that MoaA accelerates the 3',8-cyclization by 6 ~ 9 orders of magnitude. The rate acceleration was achieved by a combination of partial restraints of GTP conformation and transition state stabilization by the R17 residues. To our knowledge, this is the first insights into the magnitude and mechanism of rate acceleration of a radical reaction catalyzed by a radical SAM enzyme.

Methods:

General.

Sodium dithionite (SDT), L-methionine, 2-amino-6,7-dimethyl-4-hydroxypteridine (dimethylpterin, DMPT), 5'-deoxyadenosine (5'-dA), QAE Sephadex A-25 resin, C18

reverse-phase Silica 90 resin were purchased from Sigma-Aldrich, β -mercaptoethanol (β ME) from Calbiochem, dithiothreitol (DTT) from Amresco, Guanosine 5'-triphosphate (GTP) from Chem-Impex, Amberlite CG-50 resin from Fisher Scientific, G-25 Sephadex resin from GE Healthcare, Biogel-P2 size exclusion column from Bio-Rad, Ni-NTA agarose resin from Qiagen. $[3'-^2\text{H}]$ GTP and isotopically labeled ATP were enzymatically synthesized as previously reported^{26–27}. All the SAM with natural isotope abundance and ^2H or ^{13}C enrichment were enzymatically synthesized from L-methionine and ATP with appropriate isotope labels. *Escherichia coli* (*E. coli*) DH5 α and BL21(DE3) competent cells were from Invitrogen. Data plotting and nonlinear least-squares fitting were carried out using GraphPad Prism 7. All anaerobic experiments were carried out in an mBraun glovebox maintained at 10 ± 2 °C with an O_2 concentration < 0.1 ppm. All anaerobic buffers were degassed on a Schlenk line and equilibrated in the glovebox for overnight. All plastic devices were evacuated in the antechamber of glovebox for overnight before use. 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 L-2300 column oven maintained at 40 °C. XBridge Amide column (Waters) was used for SAM detection and ODS Hypersil C18 column (Thermo Scientific) for 5'-dA, DMPT, and compound Z. UV-vis absorption spectra were determined using a U-3900 UV-vis ratio recording double-beam spectrometer (Hitachi). EPR spectra were collected using an EMXplus 9.5/2.7 EPR spectrometer system equipped with an In-Cavity Cryo-Free VT system (Bruker Biospin Corporation). Simulations of EPR spectra were performed by EasySpin²⁸. Kinetic fitting was performed by Global KinTek Explorer^{29–30}. All mass spectra were recorded on a 6224 accurate-mass time-of-flight mass spectrometer equipped with a Dual ESI source, and accurate mass data were obtained by internal calibration using a secondary nebulizer to continuously deliver the reference solution. Flow-injection analysis was conducted in full-scan mode using positive-ion polarity over the range of 80 ~ 1700 m/z. LCMS analysis was conducted on a 6224 TOF LC/MS system equipped with a Phenomenex Kinetix C18 EVO column (3×100 mm, 2.6 μ particle), monitored by a diode array detector (254 nm) under the following gradient: hold 100% A (100:3:0.3 H_2O :MeOH:formic acid) for 0 ~ 0.5 min, then ramp 0% ~ 100% B (100:3:0.3 MeCN: H_2O :formic acid) from 0.5 ~ 8 min, flow rate: 0.5 mL/min. NMR samples (isotopically labeled SAM) were prepared in 5 mm diameter NMR tubes with sealed capillary tube containing 0.05 wt. % 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid, sodium salt in deuterium oxide (99.9 atom % ^2H). All NMR spectra were recorded on a 700 MHz Bruker Advance III NMR spectrometer operated with TopSpin NMR software and analyzed by SpinWorks 4.2.10.

X-band CW-EPR experiments.

MoaA assays were carried out under strict anaerobic condition (< 0.1 ppm O_2). MoaA (450 μM) was first pre-reduced by 1.5 mM sodium dithionite (SDT) in buffer B (100 mM Tris HCl pH 7.6, 0.3 M NaCl, 10% glycerol) with 5 mM DTT at 25 °C for 60 min. To initiate the reaction, the pre-reduced MoaA was then mixed with SAM and GTP in buffer B. The final concentration of each component was 300 μM MoaA, 1 mM SDT, 1 mM SAM (or isotopologues) and 1 mM GTP or $[3'-^2\text{H}]$ GTP. For the EPR experiments with $[4'-^{13}\text{C}]$ SAM or $[\text{ribose-}^{13}\text{C}_5]$ SAM, a final concentration of 500 μM MoaA was used to

improve the signal-to-noise ratio. The samples were transferred into EPR tubes and freeze-quenched manually by submerging in isopentane slush bath (~ 110 K) in the glovebox. The samples were then taken out of the glovebox and kept in liquid nitrogen for X-band continuous wave EPR characterization.

The EPR spectra for the [4Fe-4S] clusters were determined at 15 K. EPR parameters were a microwave frequency of 9.36 ~ 9.39 GHz; a power of 1.589 mW; modulation amplitude of 10 G; modulation frequencies of 100 kHz; time constants of 0.01 ms and a scan time of 100 s. Quantitation of the [4Fe-4S] cluster signal was carried out using a 1 mM Cu(II) sample as standard. The Cu(II) standard was prepared from a ~ 100 mM CuSO₄ stock solution in H₂O with the Cu(II) concentration determined by light absorbance at 810 nm ($\epsilon_{810\text{nm}} = 11.8 \text{ M}^{-1}\cdot\text{cm}^{-1}$). The stock solution was diluted in 2 M NaClO₄, 10 mM HCl, 20% glycerol to prepare a 1 mM Cu(II) standard. EPR spectra of Cu(II) standard were determined under the following conditions: A microwave frequency of 9.36 ~ 9.39 GHz; a power of 0.02 mW; modulation amplitude of 4 G; modulation frequencies of 60 kHz; time constants of 0.04 ms and a scan time of 30 s.

The EPR spectra of the organic radical with non-labeled or isotopically labeled substrates were all determined at 45 K, with a microwave frequency of 9.36 ~ 9.39 GHz; a power of 12.62 mW; modulation amplitude of 2 G; modulation frequencies of 100 kHz; time constants of 0.01 ms and a scan time of 70 s. Quantitation of the organic radical signal was carried out using a 18.0 μM flavosemiquinone radical (Flv•) as standard. Flv• was prepared as previously reported^{31–32}. 1.05 eq. of flavodoxin was incubated with 0.5 eq. of SDT at 37 °C for 60 min under anaerobic condition. The concentration of Flv• was determined based on the light absorbance at 579 nm ($\epsilon_{579\text{nm}} = 4570 \text{ M}^{-1}\cdot\text{cm}^{-1}$). The sample was then transferred into EPR tube and frozen in an isopentane slush bath. EPR parameters for Flv• standard were a microwave frequency of 9.36 ~ 9.39 GHz; a power of 0.3991 μW ; modulation amplitude of 10 G; modulation frequencies of 100 kHz; time constants of 0.01 ms and a scan time of 50 s.

The recorded EPR spectrum was first processed by a baseline correction using Xenon software. For the EPR spectrum of organic radical species, the signal from [4Fe-4S] cluster was subtracted by a control reaction without SAM. The observed signal intensity was determined by calculating the double integration of the first derivative EPR spectrum. This observed intensity was then normalized by the following equation:

$$\text{normalized intensity (DI/Nc)} = \frac{\text{Observed intensity (DI)}}{M \cdot g \cdot f \cdot \sqrt{P}}$$

where M represents modulation amplitude, P is the microwave power, g is the average g value of the radical, f is a packing factor related to the inner diameter of EPR tube. The normalized intensity was compared to that of standard to get the exact spin concentration of the sample. All EPR spectral simulations were performed using the EasySpin software²⁸.

Quantitation of (4′R)-5′-dA, (4′S)-5′-dA and 3′,8-cH₂GTP by MoaA/MoaC coupled assay.

MoaA/MoaC coupled assay was carried out to study the kinetics of the MoaA-catalyzed reaction. MoaA (300 μM) was incubated with MoaC (300 μM), GTP (1 mM), SAM (1 mM), SDT (1 mM) in buffer B with 5 mM DTT and 1 mM MgCl₂. MoaA was first incubated with SDT and MoaC at 25 °C for 30 min to pre-reduce MoaA and transform all the co-purified 3′,8-cH₂GTP to cPMP. Reaction was then initiated by mixing with GTP and SAM in the same buffer. A 30 μL aliquot of the reaction was quenched with 120 μL 3% (w/v) trichloroacetic acid (TCA) at each time point. The quenched sample was centrifuged at 16,100 × g for 10 min to remove protein precipitation. To derivatize cPMP into compound Z (CmdZ)¹⁵, a solution (25 μL) containing 0.2% (w/v) I₂ and 0.4% (w/v) KI was mixed with 25 μL of the cleared reaction mixture followed by incubation at room temperature for 20 min. The sample was then diluted with mobile phase (0.1% TFA in H₂O), centrifuged to remove any precipitant, and injected onto the HPLC equipped with an ODS Hypersil column (4.6 × 250 mm). Chromatography was performed by an isocratic elution with 0.1% TFA in H₂O (0.8 mL/min, 12 min, retention time: 5.5 min) and monitored by fluorescence (excitation: 367 nm, emission: 450 nm). The concentration of CmdZ was quantified based on the standard curve from authentic CmdZ standard. CmdZ standard was prepared by following reported protocol³³. To quantify the residual unconverted 3′,8-cH₂GTP, an aliquot of quenched sample was chemically derivatized to DMPT by following a previous published method¹⁵. The quenched mixture (50 μL) was incubated at 95 °C for 5 min to completely hydrolyze MoaA product and release the 6-hydroxy-2,4,5-triaminopyrimidine moiety. After cooling down, the sample was neutralized by 5 μL 0.75 M NaOH and mixed with 15 μL of 0.66% (v/v) 2,3-butanedione in 0.9 M Tris•HCl (pH 8.5). The solution was incubated at 95 °C for 45 min. The resultant solution was then diluted with mobile phase (30 mM ammonium formate, pH 4.5), centrifuged to remove any precipitant, and injected onto the HPLC equipped with an ODS Hypersil column (4.6 × 150 mm). Chromatography was performed by an isocratic elution with 7.5% MeOH, 92.5% 30 mM ammonium formate, pH 4.5 (22.5 min, 1 mL/min, retention time: 11.5 min) and monitored by fluorescence (excitation 365 nm, emission 445 nm). The concentration of DMPT was quantified based on the standard curve of commercial DMPT standard. The amount of 3′,8-cH₂GTP was calculated as a sum of CmdZ and DMPT. The amount of co-purified 3′,8-cH₂GTP was subtracted by a MoaA/MoaC reaction with all the components except SAM analyzed by the same protocol. The remaining quenched sample was used for (4′R)-5′-dA and (4′S)-5′-dA quantitation. After removal of precipitation, the quenched sample was directly injected onto the HPLC equipped with an ODS Hypersil column (4.6 × 150 mm). Chromatography was performed by a linear gradient of 0 ~ 20% MeOH in 30 mM ammonium formate, pH 4.5 (18 min, 1 mL/min, retention time: 12.7 min for (4′S)-5′-dA, 14.6 min for (4′R)-5′-dA) and monitored by UV absorption at 260 nm. The concentration of both (4′R)-5′-dA and (4′S)-5′-dA were quantified based on the standard curve of commercial (4′R)-5′-dA standard.

Isolation of (4′R)-5′-dA, (4′S)-5′-dA from large scale MoaA reaction.

MoaA/MoaC coupled assay (5 mL) was incubated at 25 °C for 3 h. Reaction system was quenched by 25% TCA, centrifuged to remove precipitation and neutralized to pH ~ 5. The sample was first loaded onto 15 mL C18-silica column which was equilibrated in H₂O. The

column was first washed with 150 mL H₂O, followed by a linear gradient (300 + 300 mL) of 0 ~ 30% MeOH in H₂O to elute the products. Fractions containing desired compounds were monitored by UV absorption at 260 nm and HPLC. (4' *R*)- and (4' *S*)-5'-dA can be well separated at this step. Fractions with pure epimer were pooled, concentrated by rotary evaporation, and further purified by HPLC using conditions same as those for 5'-dA quantitation. Both isolated products were lyophilized and characterized by ¹H NMR and ¹H-¹H COSY.

DFT calculation.

The hybrid functional Becke³⁴, 3-parameter, Lee-Yang-Parr³⁵ with the D3 version of Grimme dispersion with Becke-Johnson damping³⁶ (B3LYP-GD3BJ) and the 6-311G(d) basis set³⁷ were applied to all calculations in the Gaussian 16 program³⁸. The geometric structures were optimized at gas phase for all reactants, products and transition states in eight systems. These are (1) GTP-C3'• (no restraints): the GTP-C3'• without any frozen atoms; (2) GTP-C3'• (triphosphate restraints): the GTP-C3'•, in which the backbone atoms of triphosphate group (PG, O3B, PB, O3A, PA) were frozen; (3) GTP-C3'• (with restraints): the backbone atoms of triphosphate group (PG, O3B, PB, O3A, PA) and three atoms of purine group (O6, N1, N2) were frozen; (4) GTP-C3'•/R266/R268 (with restraints): the GTP-C3'• and two positively charged Arg residues (R266 and R268, truncated at the delta carbon), in which the restraints on the GTP-C3'• are the same as the third system and all heavy atoms in the truncated Arg residues were frozen; (5) GTP-C3'•/R17(+)/R266/R268 (with restraints): the GTP-C3'• and three positively charged Arg residues (truncated at the delta carbon, all heavy atoms frozen), in which the restraints on the GTP-C3'• are the same as the third system; (6) GTP-C3'•/R17(0)/R266/R268 (with restraints): the GTP-C3'• and three Arg residues, with the same restraints as the fifth system except that positive charge of R17 was removed. (7) GTP-C3'•/K17(+)/R266/R268 (with restraints): the methyl guanidium of R17 was replaced with methylamine (K17) by placing the positively charged amino group of K17 at the location identical to the carbon of guanidium group of R17; (8) GTP-C3'•/K17(0)/R266/R268 (with restraints): the GTPC3'• with the same amino acids and restraints as the seventh system except that positive charge of K17 was removed. The heavy atoms in the truncated Arg residues were frozen as a part of the enzyme. As the inclusion of all residues near the GTPC3'• would increase greatly the computational cost, the backbone atoms of triphosphate group were frozen to mimic the restraints imposed on the triphosphate group by enzyme through many hydrogen bonds. In addition, the O6 atom of purine group was frozen because of the restraints from R266 and R268, and the N1 and N2 atoms were frozen to mimic the restraints from the [4Fe-4S] cluster. The vibrational frequencies for all structures were calculated at 298.15 K and 1 atm pressure to confirm the reactants or products, which have no imaginary frequency, and the transition states, which have only one imaginary frequency. The nature of the transition state was verified by the vibrational mode of the imaginary frequency as well. The thermal correction to Gibbs free energy was also acquired from the frequency analysis. The intrinsic reaction coordinate (IRC)³⁹ calculations on transition states were performed to confirm the appropriate connected reactants and products. The correct radicals on C-3' in reactants and on N-7 in products were verified from the calculated spin densities. All geometric structures shown in the figures were generated by PyMOL.

Results:

Observation of an organic radical species in MoaA reaction.

To investigate the mechanism of MoaA catalysis, we characterized wt-MoaA using X-band EPR spectroscopy. The anaerobically purified and reconstituted MoaA carried 5.8 ± 0.6 Fe per monomer, corresponding to 1.45 ± 0.15 eq. of [4Fe-4S] cluster per monomer. In the absence of substrates, the EPR spectrum of MoaA at 15 K showed two axial signals (Figure S1A). The ratio of the two signals and the total spin concentration were not affected by the presence of SAM, but the presence of GTP slightly increased the amount of reduced clusters (from 0.94 ± 0.09 eq. to 1.11 ± 0.06 eq. per monomer) and significantly increased the population of the wider signal (Figure S1B and S1C, Table S1). The two signals were not affected by the nature of the buffer systems (Figure S2), and thus the observations suggest the presence of two [4Fe-4S]⁺ cluster species. The nature of these two species is currently unknown.

Remarkably, in the presence of both GTP and SAM, an additional feature at $g = 2.0$ was observed (Figures 2A and S1D). When the EPR spectra were determined between 15 ~ 90 K, the new feature became better resolved as the temperature increases from 15 to 45 K (Figure 2A). The intensity of this signal decreased at temperatures above 55 K. The observed temperature dependent behavior of this signal suggests that it is likely an organic radical with a fast spin relaxation property.

At 45 K, the spectrum had approximately ~ 100 G width centered at $g = 2.0$, consistent with an organic radical (Figure 2B, black trace 1). Intriguingly, we observed a distinct EPR spectrum when [3'-²H]GTP was used as substrate (Figure 2C, black trace 1). We hereby tentatively designate the signal observed with GTP with natural isotope abundance as X, and the signal with [3'-²H]GTP as Y. The spectrum of radical X was simulated with hyperfine coupling to three $S = 1/2$ nuclei, suggesting the presence of three protons adjacent to the radical (Figure 2B, red trace 1, Table S2). The radical Y spectrum was simulated by parameters identical to X, but with one of the three $S = 1/2$ nuclei replaced with a $S = 1$ nucleus (Figure 2C, red trace 1, Table S2). These observations suggest that X and Y are the same radical with different hyperfine splitting patterns due to the replacement of one of the three ¹H with ²H.

The observed organic radical exhibits a unique relaxation property.

In general, organic radicals are best characterized at ~ 77 K. However, the radicals X and Y were broadened and less intense at 77 K than at 45 K, indicating its fast relaxation property. Thus, radical X was characterized by microwave power saturation experiments. When the radical signal intensities were quantified and plotted against the root square of microwave power, no apparent signal saturation was observed up to 16 mW and only a partial saturation was observed even at 200 mW. The resulting data were analyzed by a non-linear curve fit to the following equation⁴⁰ (Figure S3):

$$\text{signal intensity} = \frac{K \times \sqrt{P}}{[1 + (P/P_{1/2})]^{0.5b}} \quad (\text{eq. 1})$$

where K power in mW, $P_{1/2}$ is the microwave power at half saturation, and b is the inhomogeneity parameter⁴¹. The analysis revealed $P_{1/2} = 34.5 \pm 10.8$ mW with $b = 0.68 \pm 0.08$. The observed $P_{1/2}$ was significantly larger than those reported for organic radical without any other paramagnetic centers. In the radical SAM enzyme PolH, the substrate radical trapped in a C209A mutant enzyme active site exhibited $P_{1/2} = 23.3 \pm 17.7$ μ W at 77 K (Figure S4)⁴². Similarly, characterization of a substrate radical observed in Cfr requires a low microwave power (20 μ W) to avoid saturation⁴³. In BtrN, another member belongs to SPASM/Twitch family, a substrate radical was characterized with a microwave power of 400 μ W⁴⁴ and both [4Fe-4S] clusters in the active site were proposed to be 2+ state when the radical was formed⁴⁵. Therefore, the relaxation property of the radical in MoaA is faster than any other radicals observed in radical SAM enzymes, and suggests the presence of another paramagnetic center in the active site, most likely the reduced auxiliary [4Fe-4S] cluster.

Characterization of the radical using isotopically labeled SAM.

The hyperfine splitting pattern of signal X suggested the presence of three protons adjacent to the radical, which eliminates the possibility of GTP C-3' or N-7 radical. Thus, we investigated the possibility of a radical on 5'-dA using isotopically labeled SAM. SAM isotopologues were synthesized enzymatically from L-methionine and site-specifically isotope labeled ATP using recombinant SAM synthetase. The isotope labeled ATP was prepared from either glucose or ribose with corresponding isotope labeling using de novo purine biosynthetic enzymes²⁶⁻²⁷. To investigate if the radical is indeed on 5'-dA, we first characterized radical X using [ribose-¹³C₅]SAM. The resulting EPR spectrum showed significantly broader features with ~ 200 G width (Figure 2B trace 2) with a large hyperfine coupling constant (~ 200 MHz, Table S2) consistent with a hyperfine coupling with ¹³C. These results suggest that radical X is on ribose carbon(s) of 5'-dA.

To determine the exact location of radical X, we prepared SAM isotopologues with either ¹³C or ²H on 4' - or 5' -position. When [5'-¹³C]SAM was used in the reaction, only a small hyperfine coupling constant (~ 30 MHz) derived from the 5'-¹³C labeling was observed (Figure 2B trace 3, Table S2), indicating that the radical was not on C-5'. When [5'-²H₂]SAM was used, two of the three major hyperfine splitting features in the EPR spectrum of radical X collapsed, resulting in a signal with only one apparent hyperfine splitting (Figure 2B trace 4). The simulation revealed that the radical was hyperfine coupled to only one $S = 1/2$ nucleus (Table S2). These observations suggest that radical X is hyperfine coupled to H-5' of SAM. When [4'-¹³C]SAM was used, the EPR spectrum of the radical exhibited a large hyperfine splitting (~ 200 MHz) comparable to that observed in the reaction with [ribose-¹³C₅]SAM (Figure 2B trace 5, Table S2). These observations are most consistent with radical X being a 5'-deoxyadenos-4'-yl radical (5'-dAC4'•).

The radical Y was also characterized using the isotopically labeled SAM, which paralleled the observations with X (Figure 2C, Table S2). While [ribose-¹³C₅]SAM caused a large hyperfine splitting (~ 200 MHz), [5'-¹³C]SAM resulted in a small hyperfine splitting (~ 30 MHz), indicating that radical Y is not on C-5'. When [5'-²H₂]SAM was used, the original two major hyperfine splitting features both collapsed, resulting in a singlet signal without

any defined hyperfine splitting. When $[4' -^{13}\text{C}]\text{SAM}$ was used, a large hyperfine splitting (~ 200 MHz) comparable to that with $[\text{ribose-}^{13}\text{C}_5]\text{SAM}$ was observed. All these observations suggest that radical Y is also on C-4'. Since the ^1H or ^2H -atom at the 3'-position of GTP is transferred to the 5'-position of 5'-dA during the catalysis, the presence of hyperfine splitting with only two protons in radical Y can be explained by the presence of one ^2H at the 5'-position of 5'-dA. On the basis of these considerations, we propose radical Y as $[5' -^2\text{H}]5' -\text{dA-C4}'\bullet$.

Detection of (4' S)-5'-deoxyadenosine ((4' S)-5'-dA) formation.

Based on the observation of $5' -\text{dA-C4}'\bullet$, we sought for shunt products that may be derived from $5' -\text{dA-C4}'\bullet$. When MoaA reaction was quenched by acid and analyzed by HPLC, we observed a small peak with retention time ~ 2 min earlier than 5'-dA formed from SAM cleavage (Figure 3A). The UV absorption spectrum of this peak was consistent with an adenine-containing molecule. This minor product was observed in both MoaA reaction and MoaA/MoaC coupled reaction in similar quantity (Figure 3A).

To confirm its identity, this minor species was isolated from a large scale MoaA reaction and characterized by MS and NMR. The LCMS analysis of the purified minor species and 5'-dA showed an identical molecular weight (Figure 3B). The ^1H NMR spectrum showed two aromatic signals and five aliphatic signals including the methyl signal at 1.3 ppm (compare Figure S5 and S7). ^1H - ^1H COSY spectrum (Figure S6) demonstrated connectivity of all the aliphatic ^1H signals, consistent with 5'-deoxyfuranose structure. While the overall ^1H NMR spectrum was very similar to that of 5'-dA, a significant down field shift was observed for the H-4' signal at 4.8 ppm compared to 4.3 ppm in 5'-dA, suggesting that the two compounds are structurally very similar with the most significant difference at the 4'-position. The small $^3\text{H}3' -\text{H}4'$ coupling constant (2.6 Hz, Figure S5) is consistent with the syn orientation of H3' and H4'. Therefore, these NMR data are all consistent with (4' S)-5'-dA. Together with the molecular weight determined by MS, the close migration of this species with (4' R)-5'-dA on HPLC, and the fact that it is derived from $5' -\text{dA-C4}'\bullet$ (based on the isotope labeling and kinetic analyses described below), we propose this minor species as (4' S)-5'-dA.

When MoaA reaction was carried out with $[3' -^2\text{H}]\text{GTP}$, both (4' R)-5'-dA and (4' S)-5'-dA were found to have +1 Da higher molecular weight (Figure S9A), demonstrating that (4' S)-5'-dA was also formed after H-3' atom abstraction from GTP by $5' -\text{dA}\bullet$. When the MoaA reaction was conducted in $^2\text{H}_2\text{O}$, the molecular weights of (4' R)-5'-dA and (4' S)-5'-dA were identical to those in H_2O (Figure S9B), suggesting (4' S)-5'-dA is formed by $5' -\text{dA-C4}'\bullet$ abstracting a solvent non-exchangeable H-atom from either enzyme amino acid residues or L-methionine. While precise mechanism of the transformation of $5' -\text{dA-C4}'\bullet$ into (4' S)-5'-dA remains ambiguous, the observations described here are all consistent with the presence of a shunt pathway that yields (4' S)-5'-dA via $5' -\text{dA-C4}'\bullet$.

To further test the presence of the shunt pathway, we characterized the MoaA reaction with $[4' -^2\text{H}]\text{SAM}$. This reaction yielded $3',8\text{-cH}_2\text{GTP}$ and (4' R)-5'-dA at the rates ~ 1.5 -folds faster than the reaction with non-labeled SAM or any other isotopically labeled SAM (Figure S10). The origin of the increased catalytic rate is currently unknown. On the other

hand, the reaction with $[4' - ^2\text{H}]\text{SAM}$ did not yield detectable amounts of $5' - \text{dA-C4}'\bullet$ (Figure S11) or $(4' S) - 5' - \text{dA}$ (Figure S12). The absence of shunt pathway can be explained by a slow abstraction of $^2\text{H} - 4'$ due to a primary kinetic isotope effect. These observations, therefore, are consistent with the proposed shunt pathway.

Kinetic characterization of MoaA catalysis.

To establish a kinetic model for MoaA catalysis, the kinetics of formations of $5' - \text{dA-C4}'\bullet$, $3',8\text{-cH}_2\text{GTP}$, $(4' R) - 5' - \text{dA}$ and $(4' S) - 5' - \text{dA}$ at pre-steady state were investigated. To quantify $5' - \text{dA-C4}'\bullet$, the reactions were manually freeze-quenched, characterized by EPR, and quantified based on flavodoxin semiquinone standard^{31–32} (Figure S13). Spin quantitation using Cu(II)SO_4 as a standard resulted in essentially identical results. For accurate quantitation of chemically unstable $3',8\text{-cH}_2\text{GTP}$, MoaC was included in the assay to convert $3',8\text{-cH}_2\text{GTP}$ to cPMP, which is subsequently converted to a stable fluorescent derivative, compound Z (CmdZ)^{15, 46}. The presence of MoaC did not affect $5' - \text{dA-C4}'\bullet$ formation (Figure S14). The lag phase caused by the coupled assay was estimated to be < 0.02 s based on Storer and Cornish-Bowden's method^{47–48}. A residual amount of $3',8\text{-cH}_2\text{GTP}$, likely due to its binding to MoaA, was quantified by derivatization to dimethylpterin (DMPT)^{15, 49}. Thus, the amount of $3',8\text{-cH}_2\text{GTP}$ was a sum of CmdZ and DMPT. $(4' R) - 5' - \text{dA}$ and $(4' S) - 5' - \text{dA}$ were quantified by HPLC with authentic $(4' R) - 5' - \text{dA}$ as a standard.

The time course of $5' - \text{dA-C4}'\bullet$ formation (Figure 4A and 4B) revealed that $5' - \text{dA-C4}'\bullet$ was formed in the first 2 min with the maximum amount of $1.6 \pm 0.3\%$ of MoaA ($4.7 \pm 1.0 \mu\text{M}$ with $300 \mu\text{M}$ MoaA). There was a small but statistically significant delay in the formation of $3',8\text{-cH}_2\text{GTP}$ relative to that of $(4' R) - 5' - \text{dA}$ in the first ~ 2 min (Figure 4A). The formation of $(4' S) - 5' - \text{dA}$ was significantly slower than that of $5' - \text{dA-C4}'\bullet$ with the lag phase in the first ~ 90 sec, but progressed over time and the amount of $(4' S) - 5' - \text{dA}$ exceeded that of $5' - \text{dA-C4}'\bullet$ after 3 min (Figure 4B).

The kinetic data of all four species were globally fit to kinetic models (Figure 4C, Figure S15), among which the shunt pathway model (Figure 4C) gave the best fit (Figure 4A and 4B, solid traces). In this model, the MoaA catalysis is consisted of two paths. The main path produces $3',8\text{-cH}_2\text{GTP}$ and $(4' R) - 5' - \text{dA}$ via $\text{GTP-C3}'\bullet$, and is represented by $\text{S} \rightarrow \text{I} \rightarrow \text{P}$, where **S** represents MoaA in complex with GTP and SAM (Michaelis complex), **I** represents $\text{GTP-C3}'\bullet$, and **P** represents $3',8\text{-cH}_2\text{GTP}$. The shunt pathway that yields $(4' S) - 5' - \text{dA}$ via $5' - \text{dAC4}'\bullet$ is represented by $\text{I} \rightarrow \text{I}' \rightarrow \text{P}'$, where **I'** represents $5' - \text{dAC4}'\bullet$, and **P'** represents the shunt product $(4' S) - 5' - \text{dA}$.

Most significantly, this analysis allowed determination of the rate constant for the transformation of $\text{GTP-C3}'\bullet$ (**I**) into $3',8\text{-cH}_2\text{GTP}$ (**P**, $2.7 \pm 0.7 \text{ s}^{-1}$). Since the aminyl radical intermediate did not accumulate at a detectable amount, the $\text{GTP-C3}'\bullet$ addition to C-8 is likely the rate determining step of this transformation, and thus represented by k_2 . The k_1 is the rate determining step of the entire reaction and consistent with the k_{cat} value determined under steady-state conditions ($0.0015 \pm 0.0001 \text{ s}^{-1}$, Figure S10). The observed k_2 is 3 orders of magnitude faster than k_1 , which is consistent with $\text{GTP-C3}'\bullet$ (**I**) not experimentally detectable. Therefore, the presence of the kinetically competitive shunt

pathway ($k_3 = 1.9 \pm 1.8 \text{ s}^{-1}$) allowed the first experimental determination of the rate constant for the kinetically masked 3',8-cyclization step. As described above, this competition between the two pathways is significantly affected in the reaction with [4'-²H]SAM, where kinetic isotope effect at the 4'-position substantially slows down the 5'-dA-C4'• formation and prioritizes the on-pathway reaction, leading to the diminished formations of 5'-dA-C4'• and (4'*S*)-5'-dA (Figures S11 and S12).

Evaluation of the catalytic rate acceleration.

To evaluate the rate constant from the kinetic fitting, we compared experimentally determined rate constants and activation energies (E_a) for intramolecular radical cyclization where alkyl radicals attack sp² or sp¹ carbon centers^{50–53} (Table S3). The examples include the 5',8-cyclization of 2'-deoxyadenosine, which is known as a cyclopurine lesion of DNA oxidative damage and has been well-characterized using model systems. The rate constant for this reaction was experimentally determined as $1.6 \times 10^5 \text{ s}^{-1}$ ⁵³. While no experimental E_a for this reaction has been reported, a theoretical value based on DFT calculation has been reported as 8.4 kcal/mol⁵³. In these examples shown in Table S3, the logarithm of the pre-exponential factors in Arrhenius equation to the base 10 (logA) falls in a relatively narrow range of 9.5 ~ 12.1.

Since 3',8-cyclization does not proceed without enzymatic catalysis, no experimental rate constant or E_a is available. Instead, we performed a DFT calculation of the 3',8-cyclization at 298 K. Calculation of the transformation of GTP-C3'• to 3',8-cyclo-GTP aminyl radical without any catalysis revealed a theoretical E_a of 24.6 kcal/mol (Figure 5A and 5B, **R1** → **TS1** → **P1**, Table S4). The DFT calculation also provided the relative entropy of the transition state with respect to the reactant ($\Delta S^\ddagger$) to be -0.00543 kcal/mol/K. This allows us to predict the theoretical pre-exponential factor (A) using Eyring-Polanyi equation^{55–56}:

$$A = \frac{ek_B T}{h} e^{\Delta S^\ddagger / R} \quad (\text{eq. 2})$$

, where e is the base of natural logarithms, k_B is the Boltzmann constant, T is the temperature, h is the Planck constant and R is the ideal gas constant. The theoretical logA was calculated as 12.0 based on the theoretical $\Delta S^\ddagger$, falling in the same range evaluated from model reactions. According to the logA range in Table S3, the E_a corresponds to rate constants of $1.1 \times 10^{-6} \sim 2.9 \times 10^{-9} \text{ s}^{-1}$. These evaluations are consistent with the absence of reported 3',8-cyclization in non-enzymatic reactions and support the chemically challenging nature of this transformation. For the MoaA-catalyzed 3',8-cyclization, based on the rate constant $k_2 = 2.7 \pm 0.7 \text{ s}^{-1}$ and logA range in Table S3, the E_a was estimated as 12.2 ~ 16.1 kcal/mol. Therefore, these analyses suggest that MoaA likely lowers E_a by 8.5 ~ 12.4 kcal/mol, and accelerates the reaction rate by 6 ~ 9 orders of magnitude.

We further investigated the effect of the active site environment on E_a of 3',8-cyclization. Conformational restraints were first applied to the structure of GTP-C3'•. The triphosphate moiety and O6, N1, and N2 atoms on guanine base were fixed based on the reported X-ray crystal structure⁴⁹ and ENDOR analysis⁵⁷ of MoaA in complex with GTP, where extensive

interactions were observed between these positions of GTP and MoaA active site amino acid residues or the auxiliary [4Fe-4S] cluster. The ribose moiety was not H-bonded to any of the amino acid residues, and thus allowed to move. When this partially fixed GTP-C3'• was used, the energy minimized reactant (**R2**) adopted a conformation with 21.8 kcal/mol higher free energy than that for **R1**. Since GTP-C3'• with a restraint only for the triphosphate moiety (**R1'**) had 7.3 kcal/mol higher free energy relative to **R1**, the guanine base constraint accounted for 14.5 kcal/mol increase in the free energy. Intriguingly, **R2** exhibited a significant pyramidalization at N9 of the guanine base with a C8-N9-C4-C1' improper dihedral angle of 151.4°, which may be compared to -176.5° and -173.9° for **R1** and **R1'**, respectively (Table S4). This N9 pyramidalization is even greater than those achievable in DNA through base pairing (157.6° ~ 166.1°)⁵⁸, suggesting a unique environment of the MoaA active site. Also, **R1'** and **R2** exhibited intramolecular H-bonding between 3'-OH and α -phosphate (Figure 5B, Tables S3 and S4), which was not present in **R1** and likely a unique consequence of the fixed triphosphate conformation.

With the N9 pyramidalization, **R2** adopts a conformation appropriate for C3'• addition to C8 with the C3'-C8 distance of 3.19 Å (Figure 5A and 5B, **R2** → **TS2** → **P2**, Table S4), which is significantly shorter than 3.54 and 3.35 Å in **R1** and **R1'**, respectively. Consequently, we observed an E_a of 19.2 kcal/mol, which is lower by 5.4 or 9.1 kcal/mol compared to the E_a without any conformational constraints (**TS1**) or with the fixed triphosphate (**TS1'**), respectively (Figure 5A). These observations suggest that the MoaA active site likely pre-organizes the GTP-C3'• conformation to accelerate the 3',8-cyclization. In the enzyme active site, the high free energy of the constrained GTP-C3'• (**R2**) is likely compensated by interactions between GTP and the active site as the binding free energy for GTP was previously determined by ITC as -7.4 ± 0.6 kcal/mol⁵⁹. These analyses suggest that MoaA forces the reactant into a high-energy conformation that is unachievable in solution to accelerate the radical cyclization reaction.

The theoretical E_a determined for the conformational constraint model, however, was still higher than the estimated E_a of 12.2 ~ 16.1 kcal/mol. When the three catalytically essential Arg residues (R17, R266, R268) were included in our calculation, the E_a was further lowered to 14.2 kcal/mol (Figure 5A and 5B, **R3** → **TS3** → **P3**, Table S4). The relative entropy of the transition state with respect to the reactant ($S^\ddagger$) in this calculation was -0.0077 kcal/mol/K, giving $\log A = 11.5$, within the expected range from the model reactions. The observed lowering of E_a is likely achieved through the formation of an H-bond or electrostatic interaction between R17 and 3'-OH (2.32 Å) in the transition state (**TS3**, Figure 5B). Such interaction should stabilize the transition state and facilitate the C3'• addition to C8. On the other hand, the conformation and H-bonding interactions in the reactant state were minimally affected by the presence of three Arg residues (compare **R2** and **R3**, Table S4). These observations suggest that R17 is likely critical for the transition state stabilization through its interaction with 3'-OH.

The catalytic function of R17 was further investigated by a combination of biochemical and theoretical analysis. DFT calculation using a model without R17 (**R3**• → **TS3**• → **P3**•, Table S4 and S5) increased the E_a to 17.7 kcal/mol (Table S4). DFT calculation with a deprotonated R17 (**R3**⁰ → **TS3**⁰ → **P3**⁰, Table S4 and S5) resulted in an E_a value (18.2

kcal/mol) comparable to that without R17 (Table S4). The effect of protonation at R17 on E_a can be explained by a significantly shorter H-bond / electrostatic interactions between 3'-OH and α -phosphate (1.80 vs 2.05 Å) and between 3'-OH and R17 (2.32 vs 2.61 Å) (Table S4). These DFT results suggest the critical role of the positive charge of R17 on lowering the E_a .

The catalytic function of R17 was then tested by activity assays of R17A- and R17K-MoaA. While R17A-MoaA was catalytically inactive, R17K-MoaA exhibited activity comparable to wt-MoaA (Figure S16), suggesting that the positive charge at this position is sufficient for the catalytic function. Intriguingly, in the R17K-MoaA reaction, formations of 5'-dA-C4'• (Figure S17) and (4'S)-5'-dA (Figure S18) were below their detection limits. These observations could be explained by one of the two mechanisms: (1) The R17K mutation changes the substrate orientation in the active site and increases the distance between GTP-C3' and 5'-dA-H4'; or (2) The R17K mutation further lowers the E_a and increases the reaction rate of 3',8-cyclization (k_2).

To investigate the effect of the R17K mutation on E_a , we performed DFT calculation of the R17K-MoaA catalysis, in which we replaced the methyl guanidinium of R17 with methyl ammonium as a model of K17. Since no structural information is available for R17K-MoaA, we assumed that K17 adopts the same conformation as R17 in wt-MoaA and placed the amino group of methyl ammonium at the position identical to the carbon atom of the guanidium in the wt-MoaA model. In this model (Figure 5B, **R4** → **TS4** → **P4**, Table S4), the reactant (**R4**) conformation was significantly altered from **R3**, where the ribose moved closer to the guanine base with more extensive pyramidalization of guanine N9 (the C8-N9-C4-C1' improper dihedral of 123.3°, Table S4). This conformation is achieved by the replacement of R17 with a sterically less hindered Lys and an H-bond interaction of 3'-OH and K17 (1.93 Å), which was not observed with R17 in **R3**. The H-bonding interaction between 3'-OH and α -phosphate was also significantly shorter than that in **R3** (1.65 vs 1.95 Å). The reactant conformation in the R17K-MoaA model is similar to that of the transition state (**TS4**), and consequently a significantly low E_a of 4.9 kcal/mol was observed (Table S4). The observed low E_a is consistent with the absence of the shunt reaction in the R17K-MoaA assay. When the DFT analysis was performed with a charge neutral K17 (**R4**⁰ → **TS4**⁰ → **P4**⁰, Table S4 and S5), the 3'-OH-K17 interaction was removed and the GTP-C3'• conformation (**R4**⁰) was comparable to that found in the wt-MoaA model without the positive charge on R17 (**R3**⁰). Consequently, a significantly higher E_a of 18.0 kcal/mol was observed, which was again comparable to the wt-MoaA model without R17 or with deprotonated R17. These observations suggest the importance of the positive charge at the position 17 in the MoaA-catalyzed 3',8-cyclization reaction.

Discussion:

Enzyme-catalyzed radical reactions have been attracting more attention due to the emergence of radical based reactions in biological systems. Previously, contributions of enzyme active site to the radical-mediated catalysis have mainly been discussed in B₁₂-dependent enzymes^{60–63} and ribonucleotide reductase^{64–67}. As one of the largest enzyme superfamily catalyzing radical-mediated reactions, radical SAM enzymes still lack the

knowledge of catalytic rate acceleration. Previous studies mainly elucidated the roles of radical SAM enzymes to be generating a radical at an appropriate position of the substrate^{68–69}, but focus little on the roles of active site residues in radical-mediated catalysis. This knowledge gap is caused by the kinetic masking of radical chemistry by the preceding slow radical initiation step. Our analysis here revealed, the first evidence that a MoaA radical SAM enzyme significantly accelerates the rate of radical chemistry using a sophisticated mechanism that involves transition state stabilization.

Our comprehensive biochemical and spectroscopic characterization of the MoaA-catalyzed reaction revealed the presence of a shunt pathway that diverges from the main pathway at GTP-C3'• and produces (4'S)-5'-dA via 5'-dA-C4'• (Figure 6). While in the main pathway, GTP-C3'• undergoes the intramolecular addition to C-8 to accomplish the 3',8-cyclization, in the shunt pathway, GTP-C3'• abstracts a H-atom from the 4'-position of 5'-dA, resulting in the formation of 5'-dA-C4'•. The resulting 5'-dA-C4'• is quenched by abstracting a solvent non-exchangeable hydrogen to yield (4'S)-5'-dA.

5'-dA-C4'• is unprecedented in radical SAM enzymes, and likely represents the orientation of 5'-dA relative to GTP-C3'• during the catalysis as well as the kinetics of subsequent 3',8-cyclization. In a model active site structure of MoaA¹⁵ (Figure 5C), the distance between GTP C-3' and SAM C-4' is even shorter than the distance between GTP C-3' and SAM C-5' (4.6 Å vs 5.4 Å). Therefore, while this model is likely still missing a significant part of the active site⁵⁹, the structure is consistent with the abstraction of H-4' of (4'R)-5'-dA by GTP-C3'•. However, this spatial arrangement is not unique among radical SAM enzymes. In the reported crystal structure of BtrN⁷⁰, the distance between C-1 of 2-deoxy-scyllo-inosamine (DOIA) and SAM C-4' is also comparable to that with SAM C-5' (3.7 Å vs 3.8 Å). The accumulation of 5'-dA-C4'• radical has never been observed in other radical SAM enzymes. Thus, the presence of the 5'-dA-C4'• accumulation and the shunt pathway should also reflect the inherently challenging nature of subsequent 3',8-cyclization. The discovery of this shunt pathway allowed us to determine the rate constant (k_2) for the radical-mediated C-C bond formation reaction as $2.7 \pm 0.7 \text{ s}^{-1}$. Although this value is ~ 2000 times faster than the preceding rate-determining step ($k_1 = 0.0012 \pm 0.00002 \text{ s}^{-1}$), it is still significantly slower than other radical-mediated intramolecular C-C bond formation reactions in solution (Table S3). This slow 3',8-cyclization allows GTPC3'• to abstract H-4' of 5'-dA which is comparable to the rate of 3',8-cyclization ($k_3 = 1.9 \pm 1.8 \text{ s}^{-1}$). Therefore, the structure and the kinetics of MoaA catalysis both contribute to the presence of the shunt pathway.

Evaluation of the determined rate constant for the radical-mediated C-C bond formation reaction suggested that MoaA accelerates this step by 6 ~ 9 orders of magnitude and lowers the E_a by 8.5 ~ 12.4 kcal/mol. This rate acceleration is achieved mostly by two mechanisms. A decrease of E_a of ~ 5.4 kcal/mol is achieved by constraining guanine and triphosphate of GTPC3'• while leaving the ribose flexible. This conformational constraint facilitates the pyramidalization of N9 of guanine base and formation of H-bond between 3'-OH and α -phosphate, and results in a reactant state GTP-C3'• conformation favorable for C3'• addition to C8. Another ~ 5 kcal/mol reduction of E_a was achieved by interaction with the three catalytically essential Arg residues. Among the three Arg residues, the positive charge

on R17 had the most significant effects (3.5 kcal/mol), which was experimentally demonstrated by the activity of R17A- and R17K-MoaA. DFT calculation suggested that the positively charged R17 interacts with 3'-OH of the substrate and stabilizes the transition state. Interestingly, the biochemical and theoretical characterization of R17K-MoaA suggested that this mutant is more efficient in 3',8-cyclization due to the ability of K17 to interact with 3'-OH even in the reactant state. These observations suggest that the MoaA active site is equipped with the mechanisms to significantly accelerate the rate of radical CC bond formation.

The role of R17 in the transition state stabilization is worth a particular notice. R17 was known to be essential for the catalytic activity of MoaA⁴⁹ and in humans, a mutation of the corresponding residue in MOCS1A, a human homolog of MoaA, causes the MoCD disease⁷¹. However, how the mutation of this residue causes the enzyme inactivation was unknown. This was particularly the case when R17 interacted minimally with GTP in the crystal structure and its mutation only moderately decreased the GTP binding⁴⁹. Thus, the study reported here provide the first mechanistic insights and testable hypothesis for the role of R17 in MoaA catalysis. Future mechanistic studies on the function of R17 is important not only to understand the catalytic mechanism of MoaA but also to understand the molecular cause of MoCD and a future development of novel MoCD therapeutics.

Our theoretical analysis also suggested that the reactant and product states of the 3',8-cyclization have almost equivalent free energy. Since 3',8-CH₂GTP formation is irreversible, these results suggest a need for a thermodynamic driving force likely provided by the subsequent aminyl radical reduction. Although the auxiliary cluster was proposed to serve as an electron donor for this transformation, no experimental evidence is currently available. Even the redox potential or redox state of the auxiliary cluster is not known. Therefore, it is notable that the redox state of the auxiliary cluster could be deduced from the spin relaxation property of 5'-dA-C4'•. Substrate radicals characterized in radical SAM enzymes usually exhibit $P_{1/2} \ll 1$ mW at 77 K. Thus, the fast relaxation property of the 5'-dA-C4'• signal is unlikely to be caused by the radical SAM [4Fe-4S]²⁺ cluster. The [4Fe-4S] clusters from the second subunit of the MoaA dimer is removed by more than 31 Å, and therefore unlikely to cause significant effects on the relaxation property. Thus, the observed effect is most likely caused by the presence of the C-terminal auxiliary cluster in the paramagnetic 1+ state. If this cluster is in the reduced form during the catalytic cycle, it may then be responsible for the reduction of the putative aminyl radical intermediate. The redox roles have been proposed for auxiliary clusters in the radical SAM enzymes in the SPASM/Twitch family^{44, 70, 72}, and the redox potentials have been reported for enzymes under resting states⁷³. However, no experimental demonstration has been made about their redox states during the catalytic cycle. Thus, the observations described here represent one of the first experimental evidence for the redox state of auxiliary cluster during the catalytic cycle of any SPASM/Twitch radical SAM enzymes.

In conclusion, we report the first determination of a radical SAM enzyme MoaA accelerates a radical-mediated C-C bond formation by 6 ~ 9 orders of magnitude by constraining the substrate conformation and stabilizing the transition state using a positive charge of R17 residue. Future studies of MoaA catalytic mechanism are important to provide mechanistic

basis on the cause of human Moco deficiency disease and understand how radical SAM enzymes catalyze chemically challenging radical transformations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

We thank Dr. Peter Silinski for assistance with the MS measurements and Dr. Benjamin G. Bobay at the Duke NMR facility for the assistance in collecting NMR data.

Funding Sources

This work was supported by the Duke University Medical Center, National Institute of General Medical Sciences R01 GM112838 (to K.Y.) and R01 GM061870-17 (to W.Y.), and National Science Foundation CHE-1900338 (to W.Y.). EPR spectrometer was supported by an Institutional Development Grant (ID 2014-IDG-1017) from the North Carolina Biotechnology Center. NMR instruments in the Duke University NMR Spectroscopy Center was purchased with support from NIH, NSF, HHMI and the North Carolina Biotechnology Center.

ABBREVIATIONS

GTP	guanosine 5'-triphosphate
SAM	S-adenosyl-L-methionine
5'-dA	5'-deoxyadenosine
5'-dA-C4'•	5'-deoxyadenos-4'-yl radical
Moco	molybdenum cofactor.

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Figure 1: Moco biosynthetic pathway and proposed mechanism of MoaA. (A) SAM reductive cleavage catalyzed by radical SAM enzymes. (B) Moco biosynthetic pathway in bacteria. (C) Proposed mechanism of radical-mediated C3'-C8 bond formation by MoaA.

Figure 2:

EPR characterization of the observed radical species. (A) Overlay of EPR spectra of MoaA under turnover conditions. The EPR spectra were determined at the temperatures shown in the figure. (B and C) Characterization of radical X and Y using isotopically labeled SAM. Shown are experimental (black traces) and simulated EPR spectra (red traces) of radicals X (B) and Y (C) generated in the presence of isotopically labeled SAM.

Figure 3:

Formation of (4'S)-5'-dA in MoaA reaction. (A) HPLC detection of (4'S)- and (4'R)-5'-dA in MoaA assays. Shown are HPLC traces monitored at 260 nm for (from the bottom to the top) authentic (4'R)-5'-dA standard, a control MoaA assay without SAM, MoaA assay quenched at 300 or 600 s, MoaA/MoaC coupled assay quenched at 300 or 600 s. (B) LCMS characterization of (4'S)- and (4'R)-5'-dA isolated from the MoaA reaction with GTP and SAM. Calculated m/z $[M+H]^+$ for 5'-dA ($C_{10}H_{13}N_5O_3$) is 252.1091. Shown are extracted ion chromatograms monitored at $m/z = 252.1091 \pm 0.0024$, and extracted mass spectrum at each retention time: (4'R)-5'-dA: 1.742 ~ 1.924 min, (4'S)-5'-dA: 1.358 ~ 1.562 min.

Figure 4:

Kinetic model of MoaA catalysis. (A and B) Time course of formations of 5'-dA-C4'•, 3',8-cH₂GTP, (4'R)-5'-dA and (4'S)-5'-dA. B is a magnified view of A focused on 5'-dA-C4'• and (4'S)-5'-dA. The solid lines are the results of global fitting (p-value of 0.063 and χ^2/DoF of 1.32) using KinTek Explorer^{29–30} and the parameters and the model shown in C. (C) Kinetic model and rate constants used to analyze the time course data in A and B. **S**, the Michaelis complex (MoaA•GTP•SAM); **I**, GTP-C3'•; **P**, 3',8-cH₂GTP and (4'R)-5'-dA; **I'**, 5'-dA-C4'•, and **P'**, (4'S)-5'-dA.

Figure 5:

DFT calculation of 3',8-cyclization and MoaA active site. (A) Reaction coordinates of C3'-C8 bond formation from GTP-C3'• (reactant, **R**) to yield 3',8-cyclo-GTP aminyl radical (product, **P**) through transition states (**TS**) under four conditions: (1) without any restraints, (1') with the triphosphate fixed based on the crystal structure of MoaA•GTP complex, (2) with the triphosphate and guanine base fixed, (3) condition 2 with three catalytically essential Arg residues (R17/R266/R268). (B) Calculated structures of the reactant (**R**; GTPC3'•), transition state (**TS**), and product (**P**; 3',8-cyclo-GTP aminyl radical) without restraints (1), with fixed triphosphate and guanine (2), with R17/R266/R268 (3), and with K17/R266/R268 (4). In the models 3 and 4, R266 and R268 are not shown for clarity. Key H-bonding interactions are shown with dashed lines with H-N/O distances in Å. C8 and C3' that undergo radical cyclization are highlighted with green. (C) Model of the MoaA active site¹⁵ created by overlaying the reported structures of MoaA in complex with SAM⁵⁴ (PDB ID: 1TV8, cyan) and with GTP⁴⁹ (PDB ID: 2FB3, green). Atom color: oxygens are shown in red, nitrogens in blue, phosphorus in orange, irons in brown, and sulfurs in yellow. Dashed lines indicate distances in Å between SAM C-5' and GTP C-3', between SAM C-4' and GTP C-3', and between GTP C-3' and C-8.

Figure 6:
Mechanism of MoaA catalysis with the main pathway that yields 3',8-CH₂GTP (path A), and the shunt pathway that produces (4'*S*)-5'-dA via 5'-dA-C4'• (path B). The cubes represent [4Fe-4S] clusters.