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Understanding the Role of Electron Donors in the Reaction Catalyzed by TsrM, a Cobalamin-Dependent Radical S-Adenosylmethionine Methylase

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Abstract

The cobalamin-dependent radical S-adenosylmethionine (SAM) enzyme TsrM catalyzes the methylation of C2 of L-tryptophan to form 2-methyltryptophan during the biosynthesis of thioestrepton A. Although TsrM is a member of the radical SAM superfamily, unlike all other annotated members, it does not catalyze a reductive cleavage of SAM to a 5'-deoxyadenosyl 5'-radical intermediate. In fact, it has been proposed that TsrM catalyzes its reaction through two polar nucleophilic displacements, with its cobalamin cofactor cycling directly between methylcobalamin (MeCbl) and cob(I)alamin. Nevertheless, the enzyme has been stated to require the action of a reductant, which can be satisfied by dithiothreitol. By contrast, all other annotated RS enzymes require a reductant that exhibits a much lower reduction potential, which is necessary for the reductive cleavage of SAM. Herein, we show that TsrM can catalyze multiple turnovers in the absence of any reducing agent, but only when it is pre-loaded with MeCbl. When hydroxocobalamin (OHCbl) or cob(II)alamin is bound to TsrM, a reductant is required to convert it to cob(I)alamin, which can acquire a methyl group directly from SAM. Our studies suggest that TsrM uses an external reductant to prime its reaction by converting bound OHCbl or cob(II)alamin to MeCbl, and to regenerate the MeCbl form of the cofactor upon adventitious oxidation of the cob(I)alamin intermediate to cob(II)alamin.

Keywords

cobalamin; enzyme catalysis; iron-sulfur clusters; methylation; S-adenosylmethionine; tryptophan

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INTRODUCTION

TsrM, annotated as a cobalamin-dependent radical S-adenosylmethionine (SAM) methylase, catalyzes the methylation of C2 of L-tryptophan (Trp) to form 2-methyltryptophan (MeTrp), a precursor to the quinaldic acid moiety of the thiopeptide antibiotic thiostrepton A.¹ Unlike all other characterized radical SAM (RS) enzymes, TsrM does not cleave SAM reductively to a 5'-deoxyadenosyl 5'-radical (5'-dA•).^{2, 3} In fact, it appears that the reaction is not at all radical-based. Studies by Blaszczyk et al. suggest that TsrM methylates Trp via two distinct polar S_N2 displacements, the second of which is initiated by deprotonation of N1 of Trp.⁴ The deprotonation of N1 of Trp can give rise to an electron-rich center at C2 that attacks methylcobalamin (MeCbl), which simultaneously undergoes heterolytic cleavage to transfer CH₃⁺, affording the cob(I)alamin form of the cofactor (Scheme 1). To allow for ensuing turnovers, MeCbl is regenerated via an S_N2 attack on SAM by cob(I)alamin.

The proposed mechanism of TsrM is somewhat analogous to that of methionine synthase (MetH), which catalyzes the transfer of a methyl group from methyltetrahydrofolate (MTHF) to homocysteine to form tetrahydrofolate (THF) and methionine.⁵ Upon adventitious oxidation of cob(I)alamin, MetH becomes inactive, because the resulting cob(II)alamin form of the cofactor can no longer accept a methyl group directly from MTHF to regenerate MeCbl.⁵ However, the inactive form of the enzyme can be reactivated through a one-electron reduction of cob(II)alamin by flavodoxin and a subsequent methylation of cob(I)alamin by SAM to afford MeCbl. Even under strictly anaerobic conditions, MetH is inactivated once in every 2,000 turnovers.^{6, 7}

One of the hallmarks of enzymes in the RS superfamily is the requirement of a low-potential reductant to effect the reductive cleavage of SAM to the 5'-dA•. In many RS enzymes, this requirement is satisfied by the *in vivo* protein reductants, flavodoxin or ferredoxin,^{8, 9} although it is customary to use small-molecule chemical reductants like dithionite in *in vitro* reactions. Although TsrM does not cleave SAM reductively to a 5'-dA•, it has been noted that the enzyme is significantly more active in the presence of reductants. Initial reports, which described experiments conducted with enzyme that was void of bound cobalamin upon isolation, suggested that the enzyme is most active in the presence of the mild reductant dithiothreitol (DTT).² However, later studies on TsrM isolated with almost one full equivalent of bound cobalamin indicated that the enzyme has a turnover rate that is two-fold greater in the presence of flavodoxin (flv)/flavodoxin reductase (flr)/NADPH as compared to DTT.³ Herein, we investigate the role of reducing agents with varying reduction potentials in the TsrM-catalyzed reaction, and show, as in the MetH reaction, that TsrM undergoes adventitious oxidation during turnover. The reductant is used to regenerate cob(I)alamin from cob(II)alamin or hydroxocobalamin (OHCbl), which then receives a methyl group from SAM to prime the reaction for further turnover. We have found that the low potential flavodoxin reducing system affords the greatest rate of turnover. While we still do not understand the role of the [4Fe-4S] cluster in TsrM, we hypothesize that the flavodoxin reducing system acts a direct pathway for reducing the cobalamin cofactor through use of the [4Fe-4S] cluster.

Materials and Methods

Materials.

Ferric chloride was obtained from EMD Biosciences (Gibbstown, NJ). Bradford reagent for protein concentration determination as well as the bovine serum albumin (BSA) standard were purchased from Pierce, Thermo Fisher Scientific (Rockford, IL). Dithiothreitol (DTT), L-(+)-arabinose, isopropyl β -D-1-thiogalactopyranoside (IPTG), ampicillin, spectinomycin, and kanamycin were purchased from Gold Biotechnology (St. Louis, MO). SUMO express protease was purchased from Lucigen (Middleton, WI). SAM was synthesized from ATP and L-methionine and purified as described previously.¹⁰ *E. coli* flavodoxin (flv) and flavodoxin reductase (flr) were purified as described previously.¹¹ Nick columns containing Sephadex G-50 resin were obtained from GE Healthcare (Buckinghamshire, UK). All reagents for chemical syntheses were obtained from Sigma–Aldrich unless specified further. All other materials and reagents have been previously reported,^{3, 4} or were of the highest available quality.

General Methods.

UV-vis spectra were recorded on a Cary 50 spectrometer from Varian using the WinUV software package to control the instrument. Sonic disruption of *E. coli* was conducted with a 550 sonic dismembrator (Fisher Scientific, Waltham, MA) in an anaerobic chamber (Coy, Grass Lakes, MI). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described previously.¹² Ultra-high-performance liquid chromatography with detection by tandem mass spectrometry (LC-MS/MS) was conducted on an Agilent Technologies 1200 system coupled to an Agilent Technologies 6470 QQQ mass spectrometer. The system was operated with the associated Mass Hunter software package, which was also used for data collection and analysis.

Overexpression and Purification of TsrM from *Streptomyces laurentii*.

Using methods previously described, pRham-SUMO-TsrM was overproduced in the *btuR* BL21 strain of *E. coli*, which also harbored pDB1282 and pBAD42-BtuCEDFB plasmids.¹³ Cells were cultured in LB media supplemented with hydroxocobalamin. At an OD₆₀₀ of 0.3, expression of the genes encoded on pDB1282 and pBAD42-BtuCEDFB was induced by addition of L-arabinose to a final concentration of 0.2% (w/v) while also adding FeCl₃ and L-cysteine to 25 μ M and 75 μ M (final concentrations), respectively. At an OD₆₀₀ of 0.6, the flasks were placed on ice for 20 min prior to inducing *SUMO-tsrM* expression by adding L-rhamnose to a final concentration of 0.2% (w/v) and supplementing with additional FeCl₃ and L-cysteine (final concentrations of 25 μ M and 75 μ M, respectively). Cultures were incubated at 20°C for 16 h prior to harvesting. After harvesting, cells (~50 g/16 L cell culture) were kept in liquid N₂ prior to purification.

Purification of the SUMO-tagged TsrM was performed as previously described.³ The purification of TsrM and all subsequent steps were performed in a Coy anaerobic chamber. SUMO-TsrM was purified by immobilized-metal affinity chromatography using Ni-NTA resin. Subsequently, native TsrM was generated by incubating SUMO-TsrM with 300 U of SUMO express protease (Lucigen) for 15 h. The concentration of TsrM was determined by

the Bradford assay, as has been previously described.^{3, 14} To determine the amount of bound cobalamin, TsrM was treated with 0.1 M potassium cyanide to generate its dicyanocobalamin adduct, which allowed for concentration determination by UV-vis spectroscopy ($\epsilon_{367} = 30,800 \text{ M}^{-1} \text{ cm}^{-1}$).¹⁵

Isolation of TsrM Free of Reducing Agents.

TsrM was exchanged into no-reductant buffer (50 mM HEPES, pH 7.5, 200 mM KCl, 5% glycerol) by gel-filtration chromatography using a NICK column in a Coy anaerobic chamber. The concentration of TsrM was determined spectroscopically by calculating the concentration of bound cobalamin upon treating the enzyme with potassium cyanide, as described above. TsrM was then added to a reaction containing the internal standard (IS) Tyr (0.1 mM), SAM (1 mM), and one of the following reductants: no reductant, ascorbate (1 mM), β -mercaptoethanol (1 mM), dithiothreitol (1 mM), tris (2-carboxyethyl) phosphine (1 mM), flv (25 μM)/flr (10 μM)/NADPH (1 mM). After a 10 min incubation of TsrM with SAM and reductant (when necessary), each reaction was initiated with Trp (1 mM).

Determination of TsrM Activity.

The formation of various products was quantified by LC-MS/MS as previously described.³ All reactions monitoring the activity of TsrM contained the following: 50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol, SAM (0.5 – 1 mM), Trp (0.4 – 1 mM), Tyr (100 μM), and TsrM (0.5 – 1 μM). Chemical reductants were included at 1 mM each, while the flavodoxin reducing system contained 20 μM flv, 10 μM flr, and 1 mM NADPH. Each reaction was quenched with 100 mM (final concentration) H_2SO_4 at designated time points. Standard curves for product quantification by LC-MS/MS contained 100 μM Tyr (IS) and varying concentrations of MeTrp and SAH.

Using methods similar to those previously described,^{3, 16} quenched reaction mixtures were separated on an Agilent Zorbax Eclipse-C18 RRHD column (2.1 mm $\times$ 50 mm, 1.8 μm particle size) equilibrated in 90.5% solvent A (0.1% formic acid, pH 2.6) and 9.5% solvent B (methanol). A gradient of 9.5 – 9.7% solvent B was applied from 0.1 to 0.6 min. Subsequently, a gradient of 9.7 – 35% solvent B was applied from 0.6 to 0.65 min, which was followed by a gradient from 35 – 45% solvent B from 0.65 to 1.1 min. The gradient was held constant at 45% solvent B before it was returned to 9.5% solvent B from 1.2 – 1.6 min. The column was re-equilibrated for 0.6 min before subsequent sample injections. Products were detected using Agilent Jet Stream electrospray ionization in positive mode with multiple reaction monitoring.

Quantification of NADPH Decay with TsrM under Turnover Conditions.

NADPH is the electron source for the FAD-dependent flr, which then transfers electrons to the FMN-dependent flv. By using the extinction coefficient of NADPH ($\epsilon = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm),¹⁷ the consumption of electrons can be measured quantitatively in a reaction with fld/flr/NADPH. A 900 μL reaction mixture consisting of 1 μM TsrM, 50 mM HEPES, pH 7.5, 200 mM KCl, 5% glycerol, 0.5 mM SAM, 0.1 mM Tyr (IS), 20 μM flv, 10 μM flr, and 185 μM NADPH was monitored by UV-vis spectroscopy at 340 nm. After a 10 min incubation, the reaction was initiated by adding Trp (10 μL) to a final concentration of 0.4

mM and monitored from 0 – 15 min. An identical reaction in a volume of 270 μL was run in tandem, from which 30 μL aliquots at specific time points were removed and added to H_2SO_4 (50 mM final concentration) to quench the reaction. This reaction was run in triplicate, and error bars represent the standard deviation. Product formation was quantified by LC/MS/MS as previously described.

Quantification of Flavodoxin Semiquinone with TsrM under Turnover Conditions.

The flv protein from *E. coli* binds one molecule of flavin mononucleotide (FMN), which can be reduced by one or two electrons to its semiquinone or hydroquinone forms, respectively. The one-electron reduced flv semiquinone was generated in an anaerobic chamber by incubating flv with 0.55 equivalents of sodium dithionite for 2 h at ambient temperature. The formation of flv semiquinone was apparent by the observed change in color from yellow/orange to dark blue. To remove the excess dithionite, flv semiquinone was isolated by gel-filtration with a PD-10 column. The concentration of flv semiquinone was determined by UV-vis spectroscopy using the extinction coefficient of FMN semiquinone ($\epsilon = 4570 \text{ M}^{-1} \text{ cm}^{-1}$ at 579 nm).¹⁷ A 900 μL reaction mixture consisting of 0.5 μM TsrM, 50 mM HEPES, pH 7.5, 200 mM KCl, 5% glycerol, 0.5 mM SAM, 0.1 mM Tyr (IS), and 75 μM flv semiquinone was scanned by UV-vis spectroscopy from 300 – 700 nm prior to initiating the reaction by adding Trp (12 μL) to a final concentration of 0.4 mM. The reaction was monitored and recorded at specific time points from 0 – 15 min. An identical reaction in a final volume of 210 μL was run in tandem, from which 30 μL aliquots at specific times were removed and added to H_2SO_4 (50 mM final concentration) to quench the reaction. Product formation was then quantified by LC/MS/MS as described above.

Quantification of Cobalamin by LC-MS/MS.

LC-MS/MS was used to quantify OHCbl, MeCbl, and AdoCbl in quenched reaction mixtures using a previously described method.⁴ All reactions monitoring cobalamin were performed in dim lighting and prepared as described above. Quenched reaction mixtures were separated on an Agilent Zorbax Eclipse-C18 RRHD column (2.1 mm $\times$ 50 mm, 1.8 μm particle size). OHCbl, MeCbl, and AdoCbl were detected in their +2 charge states using Agilent Jet Stream electrospray ionization in positive mode with multiple reaction monitoring. Standard curves for product quantification contained 100 μM Tyr and varying concentrations of OHCbl, MeCbl, and AdoCbl.

Generation of Premethylated TsrM.

Premethylated TsrM samples were generated by incubating 80 μM TsrM with 1 mM SAM, 40 μM flv, 20 μM flr, and 1 mM NADPH for 15 min to form MeCbl.⁴ The reaction was then exchanged into no-reductant buffer by gel-filtration using a NICK column. To protect MeCbl from degradation by photolysis, all reactions were conducted in amber tubes under dim lighting. To ensure complete isolation of TsrM from NADPH, DTT, and SAM, the final 100 μL of the protein sample was not collected from the NICK column. After the premethylated TsrM was collected, it was immediately added to a reaction containing SAM (1 mM), flv (25 μM), flr (10 μM), 0 or 1 mM NADPH, and Tyr (0.1 mM) at a final concentration of 1 – 3 μM . Each reaction was initiated with Trp (0.7 mM) and quenched in H_2SO_4 (100 mM final

concentration) at various time points as described above. Quantification of MeTrp and MeCbl by LC-MS/MS was completed as described above.

RESULTS

The Effect of Various Reducing Agents on the Activity of TsrM.

Overproduction of pRham-SUMO-TsrM was conducted in a *btuR* strain of *E. coli* along with two plasmids, as has been previously described. These plasmids include pDB1282, which encodes the *isc* operon to allow for overexpression of TsrM in the presence of iron-sulfur (Fe/S) cluster assembly proteins, and pBAD42-BtuCEDFB, which encodes the genes responsible for cobalamin uptake and transport in *E. coli*.¹³ TsrM was purified using previously described methods that result in native protein (i.e. no tags or additional amino acids) containing ~1.0 cobalamin per polypeptide.^{3, 13} Analysis of the cofactor content of our as-isolated TsrM overproduced in this manner revealed it to contain hydroxocobalamin, cob(II)alamin, and cob(I)alamin, but not MeCbl or adenosylcobalamin (AdoCbl).¹³

To probe the effect of various reducing agents on the activity of TsrM, the enzyme was incubated under turnover conditions in the presence of saturating substrates and one of the following reductants: flv/flr/NADPH, tris (2-carboxyethyl) phosphine (TCEP), DTT, β -mercaptoethanol (BME), ascorbate, or titanium (III) citrate. Samples containing TsrM, SAM, and the appropriate reductant were incubated for 10 min before initiating the reaction by adding Trp. This pre-incubation should allow for formation of MeCbl, the extent of which depends on the reductant's ability to generate cob(I)alamin. A control reaction that lacked a reductant was also conducted. Because DTT is present in the TsrM storage buffer, the enzyme was exchanged into a no-reductant buffer by anaerobic gel-filtration chromatography to ensure that the activity of the protein reflects the specific reducing agent added to each reaction mixture. As we have shown previously, protein treated in this fashion has OHCbl, cob(I)alamin, and cob(II)alamin bound, but no MeCbl or AdoCbl. The activity of TsrM in the presence of five different individual reducing agents can be seen in Figure 1. The turnover rates of TsrM with each reductant (reduction potential vs SHE at pH 7) are as follows: flv/flr/NADPH (-440 mV)¹⁸ = 6.60 min^{-1} (**red**), TCEP (-290 mV)¹⁹ = 2.84 min^{-1} (**orange**), BME (-260 mV)²⁰ = 2.57 min^{-1} (**blue**), DTT (-330 mV)²¹ = 3.03 min^{-1} (**green**), ascorbate ($+0.282\text{ mV}$)²² = 1.84 min^{-1} (**purple**), and no reductant = 1.64 min^{-1} (**black**). These data indicate that TsrM is capable of performing multiple turnovers in the absence of an external electron donor, but is most active when flv/flr/NADPH is included in the reaction. The enhanced activity of the enzyme in the presence of flv/flr/NADPH as compared to the small-molecule reductants most likely reflects the lower reduction potential of flv as well as a dedicated electron-transfer pathway from flv to the cobalamin cofactor, which would result in a higher initial concentration of bound MeCbl cofactor before addition of Trp. By contrast, the small-molecule reductants have higher reduction potentials, resulting in lower concentrations of bound MeCbl cofactor.

Role of the Flavodoxin / Flavodoxin Reductase Reducing System on TsrM Activity.

Because TsrM appears to be most active with flv/flr/NADPH, the role of this reducing system during the reaction was investigated further, with a particular goal of determining

whether reducing equivalents are consumed during each round of turnover. In the flv reducing system, NADPH transfers electrons to the FAD-dependent flr, which subsequently transfers electrons to the FMN-dependent flv. Flv, in turn, is ultimately responsible for reducing the target protein.^{24, 25} Because NADPH is the source of electrons in this system, UV-vis spectroscopy can be used to track the oxidation of this co-substrate, given that it exhibits a defined absorption feature at 340 nm ($\epsilon_{340} = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$). A reaction containing TsrM, SAM, and flv/flr/NADPH was placed in an anaerobic cuvette. The reaction was then initiated with Trp (10 μL), and the decay of NADPH was monitored over 15 min. Simultaneously, an identical reaction was conducted in an anaerobic chamber to determine the rate of MeTrp formation by LC-MS/MS. MeTrp is generated at a rate of $2.40 \mu\text{M min}^{-1}$ (Figure 2, black trace); however, NADPH decay only occurs at a rate of $0.0003 \mu\text{M min}^{-1}$ (Figure 2, red trace). These results suggest that TsrM does not consume electrons during each round of turnover.

To provide further support for our findings, the above experiment was repeated with flv semiquinone as the reducing agent in place of flv/flr/NADPH. The semiquinone form of FMN has a dark blue color, a reduction potential of -260 mV vs SHE (oxidized/semiquinone),¹⁸ and an absorption maximum at 580 nm ($\epsilon_{580} = 4,570 \text{ M}^{-1} \text{ cm}^{-1}$),^{17, 25} which allows it to be monitored and quantified. The semiquinone form of flv was generated by incubating the oxidized form of the protein with 0.55 equiv of dithionite, which forms two one-electron reducing equiv in the form of the $\bullet\text{SO}_2^-$ radical anion.^{26, 27} Following its isolation from dithionite by gel-filtration chromatography and subsequent quantification by UV-vis spectroscopy, flv semiquinone was added to a reaction containing TsrM and SAM. The reaction was initiated by adding Trp, and the concentration of flv semiquinone was monitored as a function of time. In a separate, but identical reaction mixture, aliquots of the reaction were removed and added to acid before quantifying MeTrp. MeTrp is produced at a rate of $3.8 \mu\text{M min}^{-1}$, while flavodoxin semiquinone decays at a rate of $0.06 \mu\text{M min}^{-1}$ (Figure 3). These results suggest that, in the TsrM reaction, only 1 equiv of flv semiquinone is consumed per 64 equiv of product. In conjunction with the data from the NADPH decay experiment, these results strongly suggest that TsrM does not accept external electrons during each round of turnover.

Effect of Reducing Equivalents when Using Pre-Methylated TsrM.

MetH becomes inactivated when the cob(I)alamin form of the cofactor is oxidized to the cob(II)alamin form, because cob(II)alamin cannot directly receive a methyl group from SAM.^{5, 28} In the studies described herein, TsrM is isolated with none of its bound cobalamin in the MeCbl form. In fact, the as-isolated protein contains a combination of OHCbl — wherein the cobalt ion is in the +3 oxidation state — cob(II)alamin, and a small amount of cob(I)alamin. Therefore, it seems likely that a reducing agent is required mainly to reduce OHCbl or cob(II)alamin to cob(I)alamin to allow for formation of MeCbl on the enzyme. To investigate this hypothesis, the effect of external electron donors on the activity of TsrM preloaded with MeCbl was assessed. To generate the MeCbl form of the cofactor on TsrM, the enzyme was incubated with flv/flr/NADPH and SAM for 15 min.⁴ Excess NADPH, SAM, and DTT were removed by gel-filtration chromatography using a NICK column equilibrated in no-reductant buffer. The resulting premethylated TsrM was subsequently

used in two reactions, one containing flv/flr/NADPH and one with flv/flr and no NADPH, the latter of which does not contain an external electron source. In both reactions, one containing NADPH and one lacking NADPH, TsrM displays initial rates for formation of MeTrp of $30 \mu\text{M min}^{-1}$ and $25 \mu\text{M min}^{-1}$, respectively (Figure 4A). However, in the reaction lacking NADPH, the production of MeTrp begins to level off over the course of several minutes as TsrM appears to become inactivated (Figure 4A, **red dots**). The enhancement in activity in the absence of a reducing agent observed with premethylated TsrM as compared to as-isolated TsrM suggests that the transfer of the methyl group from MeCbl to Trp does not require an external electron donor. Given that MeCbl has been shown to accumulate on the enzyme under turnover conditions,⁴ we assessed whether inactivation of premethylated TsrM via turnover in the absence of a reducing agent is accompanied by a decrease in MeCbl. LC-MS/MS was used to determine the forms of cobalamin bound to TsrM at each time point shown in Figure 4a. As has been previously reported, the concentration of MeCbl remains relatively constant in the reaction with flv/flr/NADPH.¹³ In the reaction lacking NADPH, however, there is an initial sharp decrease in the concentration of MeCbl, which we attribute to contaminating oxygen that intercepts cob(I)alamin during catalysis. This sharp decrease is then followed by a much shallower decrease in the rate of MeCbl loss. After the sharp decrease in the initial 2 min, the rate of MeCbl decay is $0.01 \mu\text{M min}^{-1}$ (Figure 5), which is similar to that of NADPH loss and flavodoxin semiquinone loss in previous experiments.

To determine if inactivation of TsrM is solely a consequence of the decrease in the MeCbl form of the cofactor, the ratio of MeTrp in solution to MeCbl on the enzyme was plotted as a function of time, which is an estimate of the formation of product per active MeCbl-bound TsrM (Figure 4b). Ideally, we would have liked to have removed aliquots of the reaction performed in the absence of reductant at each time point and then have added the aliquots to a separate reaction that contains reductant; however, the presence of the reductant would have immediately reactivated the enzyme. As can be seen in Figure 4b, when the data are normalized by the concentration of TsrM that contains bound MeCbl at each time point, the activities in the absence or in the presence of NADPH are closely aligned. To provide further evidence that the activity of MeCbl-bound TsrM is independent of NADPH, we repeated the experiments in Figure 4, which were done with $1.5 \mu\text{M}$ TsrM, with two additional concentrations of enzyme. As can be seen in Figure 6, when the experiment performed in Figure 4 is repeated with $1 \mu\text{M}$ or $3 \mu\text{M}$ TsrM, the formation of product per MeCbl-bound TsrM is again independent of NADPH.

DISCUSSION

TsrM is a clear outlier in the RS superfamily. Although it contains a [4Fe–4S] cluster that is ligated by cysteines in a canonical CxxxCxxC RS motif, it does not catalyze the reductive cleavage of SAM to a 5'-dA•. In fact, previous studies suggest that TsrM methylates C2 of Trp via a completely polar reaction that involves an $\text{S}_{\text{N}}2$ attack on SAM by cob(I)alamin to generate MeCbl, and an $\text{S}_{\text{N}}2$ attack on MeCbl by a C2 carbanion of Trp to yield MeTrp. This noncanonical behavior of an RS enzyme begs the question, what is the role of the Fe/S cluster in TsrM? The results of the study herein suggest that the role of a reductant in the TsrM reaction is two-fold. First, it is required to “prime” the system for multiple turnover.

As we determined previously,¹³ there is almost no free MeCbl in *E. coli* and most likely in *Streptomyces laurentii*, which produces TsrM. The formation of alkylcobalamins (e.g. MeCbl or AdoCbl) requires that OHCbl or cob(II)alamin be reduced to cob(I)alamin in order to acquire a methyl group directly from SAM, which primes the reaction.^{3, 4} The second role of the reductant is to repair the cofactor after adventitious oxidation to cob(II)alamin during turnover. Consistent with these roles, our results indicate that the actual transfer of the methyl group from MeCbl to Trp does not require external electrons, given that multiple turnovers can take place in their absence once the system is primed. The means by which cob(I)alamin is generated by the *in vivo* reductant, however, is not fully understood. In one scenario, flv/flr/NADPH can reduce the Fe/S cluster, which can subsequently reduce cobalamin, as is the case in the corrinoid iron-sulfur proteins.^{29, 30} If this mechanism is indeed employed by TsrM, it would explain the role of the [4Fe-4S] cluster on the enzyme, which has remained elusive. In a second scenario, flv/flr/NADPH would reduce the cobalamin cofactor directly, as is observed in Meth.¹⁸

By elucidating the role of a reducing agent in the TsrM reaction, we understand better conflicting reports about its activity in the presence of varying reductants. A study by Pierre et al. indicated that TsrM is most active when DTT is used as the reducing agent.² By contrast, Blaszczyk et al. reported TsrM to be twice as active in the presence of flv/flr/NADPH.³ However, the former study was performed with TsrM that did not contain bound cobalamin, and activity was dependent on the addition of MeCbl to the reaction mixture; whereas, the latter study was performed with cobalamin-bound TsrM. In the studies with cobalamin-free TsrM, the enzyme was more active with DTT than dithionite most likely because dithionite can bind to free-cobalamin and form an inactive six-membered form of the cofactor.³¹ Moreover, because MeCbl is directly incorporated into the enzyme, the enzyme is already in its active form. Because our cobalamin-bound TsrM is not isolated with any MeCbl, a reducing agent is necessary to reduce the various cobalamin species to cob(I)alamin, which attacks SAM to generate MeCbl.

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ABBREVIATIONS

5'-dA•	5'-deoxyadenosyl radical
β	Mercaptoethanol
DTT	dithiothreitol
flr	flavodoxin reductase
flv	flavodoxin
LC-MS/MS	liquid chromatography tandem mass spectrometry
MeCbl	methylcobalamin

MetH	methionine synthase
MeTrp	2-methyltryptophan
RS	radical <i>S</i> -adenosylmethionine
SAM	<i>S</i> -adenosylmethionine
TCEP	tris (2-carboxyethyl) phosphine
Trp	tryptophan

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Figure 1. Time-dependent formation of MeTrp by TsrM (1 μ M) in the presence of flv (20 μ M)/flr (10 μ M)/NADPH (1 mM) (red), TCEP (1 mM) (orange), BME (1 mM) (blue), DTT (1 mM) (green), ascorbate (1 mM) (purple), and no reducing agent (black), as well as SAM (1 mM) and Trp (1 mM). Error bars represent the standard deviation for three replicates.

Figure 2. Formation of MeTrp in a reaction with TsrM (1 μ M), SAM (0.5 mM), Trp (0.4 mM), flavodoxin (20 μ M), flavodoxin reductase (10 μ M), and NADPH (185 μ M) (black line). Error bars represent the standard deviation for samples run in triplicate. Decay of NADPH in TsrM-catalyzed reaction as monitored by UV-vis spectroscopy (red line).

Figure 3. Time-dependent (a) decay of flavodoxin semiquinone and (b) formation of 2-methyltryptophan by TsrM (0.5 μ M) in the presence of SAM (0.5 mM), Trp (0.4 mM), and flavodoxin semiquinone (35 μ M).

Figure 4.

(a) Time-dependent formation of MeTrp by TsrM (1.5 μM) in a reaction containing SAM (1 mM), Trp (0.5 mM), flavodoxin (25 μM), flavodoxin reductase (10 μM), and 1 mM NADPH (black) or 0 mM NADPH (red). (b) Time-dependent formation of MeTrp as a function of bound MeCbl.

Figure 5. Concentration of TsrM-bound MeCbl over time in a reaction with TsrM (1.5 μM), SAM (1 mM), Trp (0.5 mM), flv (25 μM), flr (10 μM), and 1 mM NADPH (black) or 0 mM NADPH (red).

Figure 6. Time-dependent formation of MeTrp as a function of MeCbl bound to TsrM. Reactions contained (a) 1 μM TsrM or (b) 3 μM TsrM, as well as SAM (1 mM), Trp (0.5 mM), flavodoxin (25 μM), flavodoxin reductase (10 μM), and 1 mM NADPH (black) or 0 mM NADPH (red).

Scheme 1.

Proposed mechanism for the TsrM-catalyzed reaction.