Efficient methylation of C2 in L-tryptophan by the cobalamin-dependent radical S-adenosylmethionine methylase TsrM requires an unmodified N1 amine

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Anthony J. Blaszczyk†, Bo Wang‡, Alexey Silakov§, Jackson V. Ho¶ and Squire J. Booker††‡ From the Department of Biochemistry and Molecular Biology, the Department of Chemistry, and the Howard Hughes Medical Institute, The Pennsylvania State University, University Park, Pennsylvania 16802

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TsrM catalyzes the methylation of C2 in L-tryptophan (Trp). This reaction is the first step in the biosynthesis of the quinaldic acid moiety of the thiopeptide antibiotic thiostrepton, which exhibits potent activity against Gram-positive pathogens. TsrM is a member of the radical S-adenosylmethionine (SAM) superfamily of enzymes, but it does not catalyze the formation of 5′-deoxyadenosin-5′-yl or any other SAM-derived radical. In addition to a [4Fe–4S] cluster, TsrM contains a cobalamin cofactor that serves as an intermediate methyl carrier in its reaction. However, how this cofactor donates a methyl moiety to the Trp substrate is unknown. Here, we showed that the unmodified N1 position of Trp is important for turnover and that 1-thia-Trp and 1-oxa-Trp serve as competitive inhibitors. We also showed that β-cyclopropyl-Trp undergoes C2 methylation in the absence of cyclopropyl ring opening, disfavoring mechanisms that involve unpaired electron density at C3 of the indole ring. Moreover, we showed that all other indole-substituted analogs of Trp undergo methylation at varying but measurable rates and that the analog 7-aza-Trp, which is expected to temper the nucleophilicity of C2 in Trp, is a very poor substrate. Last, no formation of cob(II)alamin or substrate radicals was observed during the reaction with Trp or any molecule within a tested panel of Trp analogs. In summary, our results are most consistent with a mechanism that involves two polar nucleophilic displacements, the second of which requires deprotonation of the indole nitrogen in Trp during its attack on methylcobalamin.

Clinical use in humans is hampered by its poor solubility and poor gastric absorption (6). Therefore, efforts are under way to alter its pharmacokinetic properties through both chemical and biosynthetic processes (7, 8).

Thiostrepton is composed of a ribosomally produced peptide of 17 amino acids that is extensively elaborated post-translationally to contain multiple thiazole and dehydroalanine residues and a central six-member nitrogen-containing heterocycle, among other modifications. In addition to these features, which are common to thiopeptide natural products, it contains a quinaldic acid moiety, essential for its antibiotic effects, that bridges two regions of the molecule to afford a second, smaller ring system. The quinaldic acid moiety is synthesized in several enzymatic steps, the first of which is the TsrM-catalyzed methylation of C2 in the indole ring of Trp (Fig. 1) (2–4).

TsrM, a radical S-adenosylmethionine (SAM)3 methylase, is a unique member of the radical SAM (RS) superfamily. Unlike other characterized members, it does not catalyze a reductive cleavage of SAM to form 5′-deoxyadenosin-5′-yl (5′-dA) or any other type of SAM-derived radical (9–11). In fact, the only SAM-related product of the TsrM reaction is S-adenosylhomocysteine (SAH), the typical byproduct of SAM-dependent methyltransferase reactions that take place via polar S–2 mechanisms (12, 13). TsrM is a member of the class B RS methylase subfamily, which require a cobalamin cofactor for turnover. These methylases have been understudied, however, primarily because of their insolubility upon overproduction in Escherichia coli (10, 14). With the exception of TsrM, ThnK, and now PoyC and CysS (15–17), the only class B RS methylases to be resolubilized from inclusion bodies, folded, and reconstituted with cobalamin and iron–sulfur (Fe/S) clusters (18–20). This procedure, however, led to incomplete cofactor loading, and the enzymes suffered from poor turnover. Moreover, catalysis was dependent on the addition of methylcobalamin (MeCbl) to reaction mixtures, which complicates the ability to detect cobalamin-related reaction intermediates (18–20). Herein, we have reported on our mechanistic studies of TsrM purified from the soluble fraction of E. coli cell lysate, which highlight the importance of N1 of the substrate

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This article contains Supplemental Figs. S1–S16 and Table S1.

1 Both authors contributed equally to this work.
2 A Howard Hughes Medical Institute investigator. To whom correspondence should be addressed. Tel: 814-865-8793; Fax: 814-865-5235; E-mail: squire@psu.edu.

The abbreviations used are: SAM, S-adenosylmethionine; RS, radical SAM; 5′-dA, 5′-deoxyadenosin-5′-yl; AdoCbl, adenosylcobalamin; DFT, density functional theory; SAH, S-adenosylhomocysteine; OHCbl, hydroxyocobalamin; MeCbl, methylcobalamin; 2D, two-dimensional.
Trp. Moreover, these studies suggested, unexpectedly, that the enzyme catalyzes the C2 methylation of Trp via a polar nucleophilic mechanism rather than through a mechanism involving substrate radicals. This conclusion was made based on the failure of a \( ^{1}H_{9252} \)-cyclopropyl analog of Trp to undergo ring opening and the finding that numerous analogs of Trp undergo C2 methylation at reasonable rates, whereas N1-substituted analogs are extremely poor substrates. Our results suggest that deprotonation of N1 is required to make C2 of Trp sufficiently nucleophilic to remove the methyl moiety from MeCbl.

**Results**

**Analysis of TsrM-bound cobalamin**

Overproduction and purification of TsrM was performed using methods described previously (10). These methods allow for purification of as-isolated TsrM that contains one cobalamin cofactor and nearly one full \([4Fe–4S]\) cluster per polypeptide, which enables reactions to be conducted in the absence of exogenously added cobalamin. However, in our previous studies, the various types of cobalamin forms bound to TsrM are not determined (10). In the present study, high-performance liquid chromatography with detection by triple quadrupolar mass spectrometry (LC-MS/MS) was used to quantify the amount of AdoCbl, hydroxocobalamin (OHCbl), and MeCbl present in as-isolated TsrM as well as TsrM after undergoing in vitro turnover. As-isolated TsrM contains \(<1\%\) MeCbl, 30\% OHCbl, and \(<1\%\) MeCbl (supplemental Fig. S1a). Under turnover conditions with dithionite as the reductant, the amount of AdoCbl decreases to 29\%, whereas the amount of MeCbl increases to 32\% (supplemental Fig. S1b). It is expected that AdoCbl would not play a role in the TsrM reaction but that the active site of the enzyme could accommodate it, given that the first step of the reaction is the transfer of a methyl group from SAM to cob(I)alamin. A recent X-ray crystal structure of the cobalamin-containing RS enzyme OxsB showed SAM to bind above the distal face of cobalamin, with its C5 carbon \(~7\) Å away from the cobalt ion of cobalamin (21).

**Design of a trap to assess substrate radical formation during turnover**

Very few detailed mechanistic studies of cobalamin-dependent RS methylases have been reported, and the results from the few systems that have been studied suggest that these enzymes most likely use multiple strategies to carry out their reactions. TsrM is the prototype for cobalamin-dependent RS methylases that catalyze the methylation of \(sp^2\)-hybridized carbon centers. In a mechanism suggested by Berteau and co-workers (9, 11), MeCbl undergoes homolysis to give cob(I)alamin and a methyl radical that adds to C2 of the indole ring of Trp. The resulting substrate radical then loses a proton and an electron with concomitant reduction of cob(I)alamin to cob(II)alamin (9–11). As a launching point to interrogate this proposed mechanism, we performed density functional theory (DFT) calculations to determine the spin distribution of the resulting substrate radical upon adding a methyl radical to C2 of Trp. DFT was carried out on a Trp model in the gas phase using the Gaussian09 program in conjunction with the B3LYP functional and 6–31G basis sets. Shown in Fig. 2 are the Mulliken spin populations of the proposed substrate radical. Most of the spin density (0.66 Mulliken spin population) resides at C3 of the molecule, which is consistent with previous studies of Trp radicals (22).
Use of Trp analogs to investigate the catalytic mechanism of TsrM

Figure 3. Analysis of TsrM-catalyzed product formation using β-cyclopropyl-Trp as the substrate. α–c, analysis of TsrM-catalyzed product formation using β-cyclopropyl-Trp as the substrate (α) and possible methylated products after ring opening (β) and without ring opening (γ). d, time-dependent formation of SAH by TsrM (1 mM) in the presence of 1 mM SAM and 1 mM β-cyclopropyl-Trp. e, LC-MS analysis of a reaction containing TsrM, SAM (10 mM), and β-cyclopropyl-Trp (10 mM). The substrate, β-cyclopropyl-Trp (m/z = 231.1), displays a retention time of 6.8 min, and the product, 2-methyl-β-cyclopropyl-Trp (m/z = 245.1), displays a retention time of 7.0 min. The reaction was conducted over a period of 38 h, and time points were taken at 0 min (black line), 12 h (blue line), 24 h (green line), and 38 h (red line). Aliquots of TsrM (10 μM) were added at 0, 12, and 24 h.

To assess whether TsrM catalysis proceeds via a mechanism that invokes a Trp radical intermediate, an analog of Trp containing a β-cyclopropyl group (β-cyclopropyl-Trp) (Fig. 3a) was synthesized and used in TsrM reactions under turnover conditions. Historically, cyclopropyl-containing substrates have been used as radical clocks to ascertain the involvement of radicals in catalysis as well as to determine their lifetimes. Cyclopropyl carbinyl radicals have been shown to undergo ring opening with a first-order rate constant of ~1.3 × 10^8 s^−1 (23), and those with aryl-containing groups, such as the indole ring of Trp, undergo ring opening with first-order rate constants on the order of 1–4 × 10^10 s^−1 (24). In a reaction containing TsrM (1 μM), SAM (1 mM), and β-cyclopropyl-Trp (1 mM), SAH, a coproduct of the TsrM-catalyzed reaction, was generated with a rate constant of 1.2 min^−1 (Fig. 3d), which is 8.3-fold slower than that of the reaction containing the normal substrate (10 min^−1). LC-MS analysis of the reaction shows peaks at 6.8 and 7.0 min that have m/z values of 231.1 and 245.1 (Fig. 3e), respectively, consistent with the β-cyclopropyl-Trp substrate and its methylated product. However, the fragmentation pattern of the methylated product could not establish its structure unambiguously, because the mass of a product with both an open (Fig. 3b) and a closed ring (Fig. 3c) would be identical if the ring-open species gains a proton and an electron.

To generate sufficient product for structural analysis by NMR, a 1-ml reaction containing 10 mM β-cyclopropyl-Trp was prepared, to which 10 μM TsrM was added once every 12 h. LC-MS analysis indicated that after 38 h nearly all of the substrate was converted to product (Fig. 3e). The reaction was then quenched, and 1.26 mg of the methylated product was obtained after purification by HPLC (supplemental Fig. S3). ^1H-NMR and ^13C-NMR spectra, as well as 2D-NMR spectra, were obtained to elucidate the chemical structure of the methylated product. As can be seen in the ^1H-NMR spectrum shown in Fig. 4, the chemical shift of the methylene protons of the cyclopropyl group after methylation moves only slightly downfield and maintains an identical splitting pattern as compared with that of the substrate, which indicates that >95% (limit of detection) of the methylated product retains the β-cyclopropyl group (individual spectra shown in supplemental Figs. S4 and S5). The 2D-NMR spectra (supplemental Figs. S6–S8), as summarized in Fig. 5, allowed the assignment of every peak to the structure of 2-methyl-β-cyclopropyl-Trp (Fig. 5a) as follows. In the COSY spectrum (Fig. 5b), correlations between H_1 ~ H_4 (Fig. 5a) on the cyclopropyl group are observed. Further, two sets of correlations in the HSQC spectrum (Fig. 5c) and long-distance correlations among H_1, H_9, and the α-carbon in the HMBC spectrum (Fig. 5d), along with the correlations observed in the COSY spectrum, also indicate that the protons on the cyclopropyl group remain in the same spin system. Signals from a second methyl group around 2.0 ppm and an additional olefin proton in the aromatic region of the proton NMR spectrum were not observed, which would be expected in a product that underwent ring opening (Fig. 3b). The retention of the cyclopropyl group on the methylated product indicates that either the methyltryptophan radical is too short-lived to initiate opening of the cyclopropyl ring, or that methylation of C2 of Trp occurs via a different mechanism. Alternative mechanisms, such as hydrogen atom abstraction from N1 of Trp, are similarly addressed by this study, because maximum unpaired electron density would also be located at C3 of β-cyclopropyl-Trp (21).
Substrate analogs containing N1 substitutions

An alternative scenario that is consistent with our observations is a non-radical mechanism whereby the π electrons at C2 perform a nucleophilic attack on the methyl group of MeCbl with the concomitant transfer of a methyl cation. In fact, it is well-known that, although C3 is the most nucleophilic site on
the indole side chain of Trp, C2 is the second most nucleophilic and even the carbons on the benzene ring are nucleophilic (25, 26). The major driving force for this nucleophilic attack in the TsrM reaction would be deprotonation of the N1 of Trp in the transition state of the reaction. Consistent with this proposal, studies by Benjdia et al. (9) show that TsrM can bind 1-methyl-Trp, as evidenced by its ability to inhibit Trp methylation, but cannot transfer a methyl group to C2 of this substrate analog.

To investigate the role of proton abstraction from N1 during catalysis, we synthesized two Trp analogs in which the nitrogen atom of the indole ring is replaced with sulfur or oxygen: 2-amino-3-(benzo[β]thiophen-3-yl)propanoic acid or 1-thia-Trp; and 2-amino-3-(benzofuran-3-yl)propanoic acid and 1-oxa-Trp, respectively (Fig. 6, group V) (NMR spectra are shown in supplemental Figs. S9 and S10). When 10 μM TsrM was incubated with SAM and 1-thia-Trp or 1-oxa-Trp, no methylated product was observed nor was SAH generated in these reactions (Fig. 7, a and b). These observations indicate...
that neither analog is a viable substrate for TsrM or that each analog is an extremely poor substrate. To set a limit of detection in these reactions, 1-oxa-Trp and 1-thia-Trp were incubated for 2 h with 120 μM TsrM. In this instance, the linear formation of product occurred with turnover numbers of 1.7 \times 10^{-3} \text{ and } 3.0 \times 10^{-4} \text{ min}^{-1}, respectively, as compared with the turnover number with Trp of 10 min^{-1}. However, it is important to note that these extremely low turnover numbers are below our reliable limit of detection for quantification of product. Nevertheless, the higher turnover number observed with 1-oxa-Trp versus 1-thia-Trp is consistent with the fact that the nucleophilicity of furan is greater than that of thiophene.

To verify that 1-thia-Trp and 1-oxa-Trp can bind to TsrM, their abilities to inhibit turnover of the normal substrate were assessed. As seen in Fig. 7, c and d, both 1-thia-Trp and 1-oxa-Trp act as competitive inhibitors with respect to Trp, exhibiting $K_i$ values of 7.3 ± 1.1 and 29.8 ± 2.8 μM, respectively. The very poor ability of TsrM to turn over 1-thia-Trp or 1-oxa-Trp, coupled with its relatively strong ability to methylate a panel of other Trp analogs (see below) (9), suggests that an unmodified N1 position of Trp is important for catalysis and likely plays a crucial role in the mechanism.

Other analogs of tryptophan are methylated to varying extents

Similar to experiments in studies conducted by Benjdia et al. (9), we synthesized or purchased a number of indole-modified analogs of Trp to assess their abilities to serve as substrates for TsrM. These compounds contain electron-donating or electron-withdrawing substituents at C4 (group I), C5 (group II), C6 (group III), C7 (group IV), and N1 (group V). As detailed above, substitutions at N1 give rise to extremely poor turnover (Fig. 6). The trends observed in group I, II, and IV compounds are particularly informative. For example, 4-methyl-, 5-methyl-, and 7-methyl-Trp all support similar activities, which are only slightly lower than that supported by Trp. Similarly, 4-fluoro- and 5-fluoro-Trp support similar activities, which are 50% of the activity supported by Trp, consistent with electron-withdrawing groups deactivating the substrate. However, electron-donating groups also appear to deactivate the substrate. For example, 5-methoxy-Trp supports a turnover number that is a little less than 50% of that supported by Trp, whereas 4-amino-Trp supports a turnover number that is about 20% of that supported by Trp. We believe that the effect of the electron-withdrawing groups is caused by the poorer nucleophilicity of C2, whereas the effect of the electron-donating groups is caused by the elevation of the $pK_a$ of the N1 nitrogen (27). Consistent with this premise, the group I and II analogs show decreasing activity with increasing electron-withdrawing strength (NO$_2$ > CN > F) as well as increasing electron-donating strength (NH$_2$ > MeO > CH$_3$). The higher activity observed with 5-nitro-Trp over that of 5-cyano-Trp might relate to a decreased electron-withdrawing ability of 5-nitro-Trp in a hydrophobic environment, especially given that resonance through the NO$_2$ group involves generation of a dianion.
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The 6-substituted analogs all support the least activity, for reasons that we do not understand, but which may be due to steric issues within the active site of the enzyme. In line with this supposition, 6-chloro-Trp exhibits very poor activity (~0.07 min⁻¹), and the activity in the presence of 6-bromo-Trp is barely detectable.

Our finding that electron-withdrawing groups deactivate the substrate is bolstered by the activity of TsrM in the presence of 7-aza-Trp, which is very low due to a resonance or tautomeric form containing electron density on N7 (supplemental Fig. S11). In fact, studies of the nucleophilicity of a variety of indoles containing simple functional groups indicate that the 7-aza-indole and 5-cyanoindole compounds are among the very least nucleophilic (28, 29). In a separate study on the use of tryptophan synthase to generate tryptophan analogs, reactions with 7-azaindole required extremely long incubation times (1 to 2 weeks) before they achieved completion (30). This observation is consistent with the mechanism of tryptophan synthase, which involves a nucleophilic attack of a C3 carbanion, with the developing charge on the N1 nitrogen of Trp in the transition state being stabilized through an electrostatic interaction with a neighboring aspartate residue. In this enzyme, deprotonation of the N1 nitrogen of indole is not essential, because the second substrate, an aminoacrylate, is a strong electrophile (31).

UV-visible spectral changes during steady-state turnover by TsrM

To investigate further the possibility of a radical-mediated mechanism, we set out to reproduce previous reports of cob(II)alamin accumulation during turnover (9, 11). The UV-visible spectrum of as-isolated TsrM is shown in Fig. 8 (black line). It exhibits a feature at 390 nm, which is consistent with the presence of cob(I)alamin. After adding SAM to this preparation, the peak at 390 nm disappears and a new peak is observed at 450 nm (Fig. 8, red line). These spectral changes are consistent with methylation of the cob(I)alamin species to afford a MeCbl species that lacks nitrogen coordination in its lower axial ligand (10). When Trp is added to this form of the enzyme under turnover conditions, no additional changes to the UV-visible spectrum from 330 to 600 nm are observed (Fig. 8, blue line), suggesting that the dominant form of the active cobalamin cofactor is MeCbl under steady-state conditions and that cob(II)alamin does not accumulate. This observation also suggests that transfer of a methyl group from methylcobalamin, rather than methylation of cob(I)alamin, limits the rate of catalysis.

Monitoring TsrM reaction under turnover conditions by EPR

EPR spectroscopy was also used to probe for cob(II)alamin accumulation during TsrM steady-state turnover. Unlike cob(I)alamin and cob(III)alamin, cob(II)alamin is paramagnetic and can therefore be detected in the absence of signals from other redox states of cobalamin, which enhances the sensitivity of the method. As we have shown previously, the EPR spectrum of TsrM in the presence of SAM reflects a five-coordinate base-off cob(II)alamin species (Fig. 9A). Upon the addition of the chemical reductant dithionite, the EPR signal at 70 K has been shown previously to disappear, which is consistent with reduction of cob(II)alamin to cob(I)alamin as well as subsequent methylation of cob(I)alamin to form MeCbl. To assess whether cob(II)alamin forms under steady-state conditions in the absence of dithionite, TsrM was premethylated as described previously, and the TsrM reaction was monitored by EPR spectroscopy. First, upon adding SAM to the as-isolated sample, the EPR spectrum displayed a significant decrease in cob(II)alamin as compared with the as-isolated sample, as observed previously (Fig. 9B). Trp was then added to the sample containing TsrM and SAM to initiate turnover, and the sample was allowed to incubate at ambient temperature for 30 s prior to loading in an EPR tube and freeze-quenching in cryogenic isopentane. Upon recording an EPR spectrum of the sample, no noticeable increase in EPR signal was observed (Fig. 9C). This behavior further suggests that cob(II)alamin does not accumulate under steady-state conditions.
One method often used to unmask transient radicals in reactions is to employ substrate analogs that exhibit enhanced radical stabilization properties, as done in the lysine 2,3-aminomutase system (32, 33), or to employ site-specific protein variants of amino acids that participate in a given radical decay pathway, as done to observe a transient substrate radical in the RS methlyase RlmN (34). To this end, reactions were conducted with 5-cyano-Trp, 6-amino-Trp, and 7-aza-Trp as substrates. The 5-cyano-Trp and 7-aza-Trp analogs are particularly suited to stabilizing a radical formed by hydrogen abstraction from N1, and 6-aminoTrp is particularly suited to stabilizing a radical formed at C3 of Trp upon the addition of a methyl radical from MeCbl. The experiments were done as described above, but the reactions were quenched after a longer incubation time to ensure that steady state had been achieved. Similar to what was observed with Trp, no substrate radical or cob(II)alamin accumulation was observed by EPR (supplemental Fig. S12).

Discussion

The reaction catalyzed by TsM is unusual for an enzyme in the RS superfamily, almost all of which use a 5′-dA to abstract a substrate-derived hydrogen atom. In previous studies of TsM by the Bertue laboratory (9, 11), which were conducted with enzyme that required the addition of MeCbl to reaction mixtures for turnover, an accumulation of cob(II)alamin was reported. This conclusion was based on UV-visible analyses during turnover, wherein a slight increase in absorbance between 400 and 476 nm and a very slight decrease in absorbance at 520 nm was observed (9). However, these changes are reminiscent of free methylcobalamin in solution, which has an absorption maximum at 520 nm, binding to TsM and perturbing the axial cobalt–nitrogen bond. This change in binding mode would result in a blue-shift in the absorbance of MeCbl to 440 nm (35). This observation, as well as the absence of 5′-dA formation during turnover, led to the suggestion that MeCbl might undergo a homolytic cleavage similar to that observed in adenosylcobalamin (AdoCbl)–dependent enzymes, to generate a methyl radical that adds to C2 of the indole ring of Trp (supplemental Fig. S1). The resulting substrate radical intermediate then loses the C2 proton and an electron to yield the methylated product (9, 11). This mechanism, however, evokes concerns. One concern derives from the observation that the formation of 2-methyltryptophan from SAM takes place with a net retention of configuration. This stereochemical outcome has been rationalized by invoking two methyl transfers (one from SAM to cobalamin and one from MeCbl to Trp) that each takes place with an inversion of stereochemistry (9, 11, 36). Homolysis of MeCbl, however, is expected to be accompanied by racemization of the methyl radical before it attacks the π system of the substrate, unless perhaps substrate radical formation is coupled to homolysis via a tight transition state. However, this reaction has no precedent in the biological or chemical literature. Secondly, it is unlikely that sufficient binding energy can be elicited from MeCbl to catalyze its homolysis. The homolytic bond dissociation energy of the Co–C bond of MeCbl (37 kcal/mol) is 6 kcal/mol higher than that of AdoCbl (31 kcal/mol) (37), and the larger size and presence of functional groups on the 5′-deoxyadenosine moiety of AdoCbl as compared with the methyl moiety of MeCbl would be expected to contribute substantially to the binding energy required for cleaving the AdoCbl Co–C bond. Although it is still not clear whether the rate enhancement for Co–C bond homolysis derives predominantly from ground-state destabilization via distortion of the cobalamin cofactor or electrostatics, the interactions between the ribose ring of AdoCbl and the polypeptide of AdoCbl-dependent enzymes appear to be critical (38). In fact, Martin and Finke (39) contend that the Co–C bond in MeCbl is strong enough to argue convincingly against any simple homolysis mechanism in MeCbl-dependent enzymes. They suggest that a one-electron reduction of methylcobalamin could weaken the Co–C bond down to ∼12 kcal/mol, providing an alternative mechanism for cleaving this organometallic bond. However, the very low redox potentials of alkylcobalamins (−1.2 to −1.6 V versus saturated calomel electrode) would seem to make these mechanisms unrealistic.

Although our studies may not unequivocally rule out the previously proposed mechanism (supplemental Fig. S2), they are most in line with a polar mechanism for the methylation of Trp in which deprotonation of N1, or a strong electrostatic interaction between a positively charged N1 and a negatively charged species in the transition state of the reaction, is essential for generating a suitably potent C2 nucleophile for attack on MeCbl. This conclusion derives from the following findings in this work: (i) the β-cyclopropyl-Trp substrate is transformed to the methylated product without ring opening; (ii) N1-substituted analogs of Trp have no or very poor turnover, whereas all other tested analogs of Trp are decent substrates, many of which are better able to stabilize radical intermediates; (iii) no observable formation of cob(II)alamin or a substrate radical is seen under turnover conditions, even with substrate analogs that exhibit strong radical-stabilizing properties; and (iv) TsM displays very poor activity with 7-aza-Trp, which is known to decrease the nucleophilicity of the indole ring of Trp.

The previously proposed radical mechanism for TsM was predicated on the observation of what was believed to be cob(II)alamin accumulation during steady state, which we were unable to reproduce. Furthermore, there is no compelling reason why an enzyme operating by a radical mechanism should be unable to efficiently catalyze product formation when 1-oxa-Trp and 1-thia-Trp are used as substrates, and use 7-aza-Trp very sluggishly. In fact, in recent studies of the RS enzyme NosL, which catalyzes the formation of 3-methylindolyl acid from Trp by a radical mechanism during the biosynthesis of nosiheptide, both 1-oxa-Trp and 1-thia-Trp served as substrates. Mechanistic studies on NosL are most consistent with the formation of a 3-methylindolin 3-yl species, which is stabilized via resonance throughout the indole moiety, including the nitrogen atom (40, 41).

The formation of MeCbl is widely accepted as occurring via an S,2 attack of cob(II)alamin on the methyl group of SAM. We propose an alternative mechanism for the transfer of the methyl group from MeCbl to Trp that we believe is more consistent with our observations (Fig. 10a). In the initial step, the deprotonation of N1 activates C2 of Trp, inducing the N1 π electrons to attack the methyl group of MeCbl via a polar nucleophilic mechanism to generate cob(II)alamin and a methylated Trp intermediate. The final product is generated by C2 proton removal and reprotonation of N1. This proposed mechanism is
similar to that of other cobalamin-dependent methyltransferases that proceed by two separate $S_n2$ displacements (35, 42).

In an alternative mechanism, which is also consistent with our findings about the significance of N1, hydrogen atom abstraction from N1 generates a C2 radical that attacks the methyl moiety of MeCbl, affording cob(II)alamin and a methylated intermediate that tautomerizes to give the final product (Fig. 10b). However, we disfavor this mechanism for several reasons. First, no radical generator has been identified in the TsrM reaction; 5'-dA is neither generated stoichiometrically with product nor in amounts that approach the concentration of enzyme in our assays. In other words, a reversible radical generator that is derived from a tightly bound SAM molecule or a derivative of it is also unlikely in the TsrM reaction (10, 11). Second, hydrogen atom abstraction from N1 would result in significant spin density at C3 of Trp, which should give rise to ring opening of the cyclopropyl group, unless methylation is exceedingly faster than ring opening. Third, the 1-oxa-Trp and 1-thia-Trp substrates appear to support turnover, albeit at extremely poor rates. Last, analogs of Trp that have radical-stabilizing properties do not give rise to observable formation of cob(II)alamin or substrate radicals, and do not enhance the rate of catalysis.

Our work on TsrM suggests that cobalamin-dependent RS methylases are not all similar and that methylation of $sp^2$-hybridized carbon centers takes place via mechanisms that are distinct from those that govern methylation of $sp^3$-hybridized centers. In the other instances of methylation of $sp^2$-hybridized carbon centers by class B RS methylases, a similar pyrrole-containing ring system is observed, which also supports the importance of the nitrogen atom in the pyrrole ring. It is expected that these enzymes, CloN6 and CouN6, catalyze their reactions by similar mechanisms.

Experimental procedures

General methods and instruments

All UV-visible spectra were measured in an anaerobic cuvette and recorded on a Varian Cary 50 spectrometer (Agilent Technologies) using WinUV software. $S$-Adenosylmethionine was synthesized from ATP and methionine and purified as described previously (43). E. coli flavodoxin and flavodoxin reductase were also purified as described previously (44). LC-MS/MS was conducted on an Agilent Technologies system coupled to an Agilent Technologies 6410, 6460, or 6470 QQQ mass spectrometer. The system was operated with the associated MassHunter software package, which was also used for data collection and analysis. Product quantification by LC-MS/MS was done as described previously (10). NMR spectra were recorded on a Bruker AV-3-HD-500 instrument and calibrated using residual solvent peaks as internal reference. Multiplicities are recorded as: s = singlet, d = doublet, t = triplet, dd = doublet of doublets, and m = multiplet. Continuous-wave EPR spectra were acquired on a Bruker ESP300 X-band spectrometer equipped with an ER 4102ST resonator. All samples were run at 70 K, and the temperature was controlled by an ER 4112-HV variable-temperature helium-flow cryostat (Oxford Instruments).

Overproduction of the tsrM gene and purification of psUMOTsrM

TsrM was overproduced and purified as described previously, and all subsequent experiments were conducted in a Coy anaerobic chamber (10). TsrM was overproduced in Rosetta-Blue (DE3) pLysS strain of E. coli cultured in M9 minimal medium that was supplemented with hydroxocobalamin and ethanolamine, which has been shown to increase cobalamin uptake (45). The strain of E. coli also contained the plasmid pDB1282 to facilitate in vivo incorporation of iron–sulfur clusters on TsrM. The purification of TsrM and all subsequent experiments were conducted in a Coy anaerobic chamber. A SUMO–TsrM fusion protein was purified by immobilized metal-affinity chromatography, and as described previously (10), native TsrM was subsequently generated by incubating the fusion protein with SUMO protease Ulp1.

Quantification of cobalamin by LC-MS/MS

Reactions quenched in 100 mM H$_2$SO$_4$ were separated on an Agilent Zorbax Extend-C18 RRHD column (2.1 mm x 50 mm,
1.8 μm particle size) that was equilibrated in 95% solvent A (0.1% formic acid, pH 2.6) and 5% solvent B (acetonitrile). A gradient of 5 - 10% acetonitrile was applied from 0 to 0.4 min, followed by the following gradients: 10 - 40% acetonitrile from 0.4 to 0.8 min, 40 - 50% acetonitrile from 0.8 to 1.6 min, 50 - 5% acetonitrile from 1.6 to 1.9 min, and re-equilibration in 5% acetonitrile from 1.9 to 2.4 min. Products were detected using Agilent Jet Stream electrospray ionization in positive mode with multiple reaction monitoring. The concentrations of AdoCbl, OHCbl, and MeCbl were determined by UV-visible spectros- copy, and a standard curve for all three molecules, which contained 62.5 nm–64 μM as well as tyrosine (the internal standard), was prepared for analysis by LC-MS/MS. AdoCbl and MeCbl were detected in their +2 charge states, having m/z values of 79.6 and 673.0, respectively. OHCbl was detected in its +2 charge state after the loss of its hydroxy group (m/z = 664.9). Supplemental Table S1 lists the retention time, parent ion, product ion, Fragmentor voltage, and collision energy used for each molecule monitored by MS. Samples for LC-MS/MS analysis were prepared as described previously (10), except that experiments were performed in the dark to prevent photolyzing of cobalamin.

Density functional theory
Geometry optimization and single-point calculations were performed using Gaussian09 Rev. A within the spin-unrestricted DFT level (46). The B3LYP functional and 6–31G basis set on all atoms was used, and all calculations were performed in the gas phase (47–49).

Synthesis of tryptophan analogs
2-(1-(1H-Indol-3-yl)cyclopropyl)-2-aminoacetic acid (β-cyclopropyl-Trp) was synthesized according to a reported procedure (50). NMR data are as follows: 1H NMR (500 MHz, D2O) δ 7.87 (d, J = 7.8 Hz, 1H), 7.36 (d, J = 8.1 Hz, 1H), 7.29 (s, 1H), 7.11 (t, J = 7.5 Hz, 1H), 7.05 (t, J = 7.4 Hz, 1H), 3.51 (s, 1H), 1.41 – 1.34 (m, 1H), 1.22 – 1.15 (m, 1H), 1.10 – 1.00 (m, 1H), 0.95 – 0.87 (m, 1H); 13C NMR (126 MHz, D2O) δ = 136.69, 127.31, 125.62, 121.28, 118.82, 118.77, 113.18, 111.09, 62.21, 18.12, 12.20, 10.75. The NMR spectrum is presented in supplemental Fig. S4.

2-Amino-3-(benzo[b]thiophen-3-yl)propanoic acid (1-thia-tryptophan) was synthesized according to a reported procedure (51), and the NMR spectrum can be seen in supplemental Fig. S9. NMR data are as follows: 1H NMR (500 MHz, D2O) δ 7.79 (d, J = 7.8 Hz, 1H), 7.81 (d, J = 7.7 Hz, 1H), 7.51 – 7.31 (m, 3H), 4.34 – 4.19 (m, 1H), 3.54 (dd, J = 15.1, 5.1 Hz, 1H), 3.37 (dd, J = 15.1, 8.3 Hz, 1H); 13C NMR (126 MHz, D2O) δ = 171.89, 140.26, 137.84, 128.53, 126.39, 124.85, 124.53, 123.21, 121.31, 52.95, 28.76.

2-Amino-3-(benzofuran-3-yl)propanoic acid (1-oxa-tryptophan) was synthesized according to a reported procedure (51). NMR data are as follows: 1H NMR (500 MHz, D2O) δ 7.766 (s, 1H), 7.61 (d, J = 7.6 Hz, 1H), 7.50 (d, J = 8.2 Hz, 1H), 7.33 (t, J = 7.3 Hz, 1H), 7.27 (t, J = 7.2 Hz, 1H), 4.28 (t, J = 6.3 Hz, 1H), 3.37 (dd, J = 15.4, 5.0 Hz, 1H), 3.29 (dd, J = 15.3, 7.3 Hz, 1H); 13C NMR (126 MHz, D2O) δ = 171.87, 155.09, 144.23, 126.80, 125.01, 123.01, 119.48, 112.95, 111.70, 52.65, 24.20. The NMR spectrum is presented in supplemental Fig. S10.

6-Amino-DL-tryptophan was synthesized according to a previously described procedure (52). NMR data are as follows: 1H NMR (500 MHz, D2O) δ 7.68 (d, J = 8.3 Hz, 1H), 7.44 (s, 1H), 7.33 (s, 1H), 7.04 (d, J = 8.3 Hz, 1H), 4.23 (t, J = 5.8 Hz, 1H), 3.42 – 3.31 (m, 2H); 13C NMR (126 MHz, D2O) δ = 172.22, 135.81, 127.15, 125.76, 124.16, 119.62, 113.97, 106.92, 106.51, 53.51, 25.66. The NMR spectra are shown in supplemental Figs. S13 and S14.

4-Amino-DL-tryptophan was synthesized using methods described previously (53). NMR data are as follows: 1H NMR (500 MHz, D2O) δ 7.55 – 7.46 (m, 1H), 7.36 (s, 1H), 7.22 – 7.13 (m, 1H), 7.04 (d, J = 7.1 Hz, 1H), 4.18 (t, J = 4.3 Hz, 1H), 3.54 – 3.44 (m, 1H), 3.40 – 3.31 (m, 1H); 13C NMR (126 MHz, D2O) δ = 173.17, 137.99, 127.32, 121.99, 121.65, 119.47, 114.79, 113.25, 104.48, 54.35, 25.83. The NMR spectra are presented in supplemental Figs. S15 and S16.

Determination of TsrM activity
All assays for monitoring the activity of TsrM under saturating concentrations of substrates contained 1 mM SAM, 1 mM Trp or 1 mM Trp analog, 100 μM Tyr (internal standard), 1–120 μM TsrM, and 1 mM dithionite. In some instances, dithionite was replaced with the flavodoxin reducing system, consisting of 25 μM flavodoxin, 10 μM flavodoxin reductase, and 1 mM NADPH. Each reaction was initiated with Trp and quenched with acid at designated time points.

Production of methylated β-cyclopropyl-Trp for analysis by NMR
The reaction mixture contained the following in a final volume of 1.4 ml: 10 mM β-cyclopropyl-Trp, 10 mM SAM, 2 mM dithionite, and 30 μM TsrM, which was added in three 10 μM increments every ~12 h. The reaction was quenched with 100 mM sulfuric acid, and the precipitated protein was removed after centrifugation. The methylated product was purified by HPLC with detection by UV-visible spectroscopy, which was conducted on an Agilent Technologies 1100 system using an Agilent diode array detector. The system was operated with the associated ChemStation software package. A Zorbax SB-C18 column (4.6 × 250 mm, 5 μm particle size) was used to separate the reaction mixtures. The column was equilibrated in solvent A (0.1% trifluoroacetic acid). A gradient of 0 – 40% solvent B (80% acetonitrile) was applied from 1 to 20 min. The percentage of solvent B was held constant from 20 to 22 min before it was returned to 0% (i.e. 100% solvent A) from 22 to 27 min. The column was then re-equilibrated in solvent A for 5 min before subsequent injections.

Assays with tryptophan analogs
L-Tryptophan, 1-methyl-L-tryptophan, 5-fluoro-L-tryptophan, 5-methoxy-DL-tryptophan, 5-methyl-DL-tryptophan, 6-methyl-DL-tryptophan, and 7-aza-DL-tryptophan were purchased from Sigma-Aldrich. 5-Cyano-DL-tryptophan was purchased from Ark Pharm, Inc., and 4-fluoro-DL-tryptophan was purchased from Gold Biotechnology; 4-methyl-DL-tryptophan, 5-nitro-DL-tryptophan, 6-fluoro-DL-tryptophan, and 7-methyl-DL-tryptophan were purchased from Santa Cruz Biotechnology. β-Cyclopropyl-DL-tryptophan, 1-thia-DL-tryptophan, 1-oxa-
Use of Trp analogs to investigate the catalytic mechanism of TsrM

dL-tryptophan, 4-amino-dL-tryptophan, and 6-amino-dL-tryptophan were synthesized as described above. All assays contained 0.5–10 μM TsrM, 1 mM SAM, 1 mM Trp analog, 100 μM tyrosine (internal standard), and the flavodoxin reducing system, consisting of 25 μM flavodoxin, 10 μM flavodoxin reductase, and 1 mM NADPH. Enzyme activity with each substrate was determined by monitoring SAH production using LC-MS/MS.

Determination of $K_i$ values for 1-modified Trp analogs

All assays contained the following: 100 μM tyrosine (IS), 0.2 μM TsrM, 500 μM SAM, 25 μM flavodoxin, 10 μM flavodoxin reductase, 1 mM NADPH, and 5–100 μM Trp. The Trp analogs examined were 1-thia-Trp and 1-oxa-Trp. The concentrations of each inhibitor were as follows: 5, 10, and 25 μM 1-thia-Trp and 50, 100, and 250 μM 1-oxa-Trp. All reactions were initiated with SAM. LC-MS/MS was used for product quantification. To obtain the type of inhibition, GraFit (Erithacus Software) was used, fitting the data globally to the following equation.

$$V = \frac{V_{\text{max}}[S]}{K_m\left(1 + \frac{I}{I^*}\right) + [S]}$$  

(Eq. 1)

Preparation of samples for UV-visible spectroscopy

TsrM (10 μM) was placed in a 1-ml anaerobic cuvette to which 400 μM SAM (6.7 μl) was added with a syringe. After 1 min, a UV-visible spectrum was acquired after the reaction, and a UV-visible spectrum was acquired after 1 min.

Preparation of EPR samples

EPR samples were prepared as described previously (10). To monitor cob(II)alamin formation under turnover conditions, a premethylated sample of TsrM was generated by incubating TsrM (70 μM) with SAM (140 μM) and dithionite (70 μM) and then exchanging the protein into a gel filtration buffer by gel-filtration chromatography to remove excess SAM and dithionite (10). The premethylated sample was concentrated to 200 μM. Samples were prepared by adding 2 mM SAM or 2 mM SAM + 2 mM Trp. Upon the addition of substrate(s), each sample was incubated at room temperature for 30 s and flash-frozen in cryogenic isopentane in an anaerobic chamber. Samples with Trp analogs were prepared as follows. 1.5 mM SAM or 1.5 mM SAM + 1.5 mM 5-cyanoTrp, 1.5 mM 6-aminoTrp, or 1.5 mM 7-azaTrp was mixed with 150 μM premethylated TsrM, incubated at room temperature for 1.5 min, and flash-frozen in cryogenic isopentane. Continuous-wave EPR was conducted as described previously (10).

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