The Thiamine Biosynthetic Enzyme ThiC Catalyzes Multiple Turnovers and Is Inhibited by S-Adenosylmethionine Metabolites*

Lauren D. Palmer, and Diana M. Downs

Department of Microbiology, University of Georgia, Athens, GA 30602

*Running title: ThiC turnover and inhibition by SAM metabolites

To whom correspondence should be addressed: Diana M. Downs, Department of Microbiology, University of Georgia, 527 Biological Sciences Building, 120 Cedar St, Athens, GA, USA, Tel.: (706) 542-7953; E-mail: dmdowns@uga.edu

Keywords: S-adenosylmethionine, radicals, thiamine biosynthesis, enzyme inhibitors, iron-sulfur protein

Background: ThiC is a radical S-adenosylmethionine (SAM) enzyme that synthesizes the thiamine pyrimidine 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P).

Results: An increase in the ThiC catalytic rate was detected when product 5'-deoxyadenosine was hydrolyzed. ThiC was inhibited by SAM metabolites.

Conclusion: ThiC is a multiple turnover enzyme and is product inhibited.

Significance: This is the first report of ThiC catalytic turnover and the identification of two SAM metabolites that inhibit ThiC activity.

ABSTRACT

ThiC (HMP-P synthase; EC 4.1.99.17) is a radical S-adenosylmethionine (SAM) enzyme that catalyzes the intramolecular rearrangement of 5-aminoimidazole ribotide (AIR) into 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P), an intermediate of thiamine pyrophosphate (coenzyme B1) biosynthesis. In this study, assay conditions were implemented that consistently generated 5-fold molar excess of HMP, demonstrating that ThiC undergoes multiple turnovers. ThiC activity was improved by in situ removal of product 5'-deoxyadenosine. The activity was inhibited by SAM metabolites S-adenosylhomocysteine, adenosine, 5'-deoxyadenosine, S-methyl-5'-thioadenosine, methionine and homocysteine. Neither adenosine nor S-methyl-5'-thioadenosine had been shown to inhibit radical SAM enzymes, suggesting that ThiC is distinct from other family members. The parameters for improved ThiC activity and turnover described here will facilitate kinetic and mechanistic analyses of ThiC.

ThiC (HMP-P synthase; EC 4.1.99.17) is a radical S-adenosylmethionine (SAM) enzyme that catalyzes the intramolecular rearrangement of 5-aminoimidazole ribotide (AIR) into 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P), an intermediate of thiamine pyrophosphate (coenzyme B1) biosynthesis. In this study, assay conditions were implemented that consistently generated 5-fold molar excess of HMP, demonstrating that ThiC undergoes multiple turnovers. ThiC activity was improved by in situ removal of product 5'-deoxyadenosine. The activity was inhibited by SAM metabolites S-adenosylhomocysteine, adenosine, 5'-deoxyadenosine, S-methyl-5'-thioadenosine, methionine and homocysteine. Neither adenosine nor S-methyl-5'-thioadenosine had been shown to inhibit radical SAM enzymes, suggesting that ThiC is distinct from other family members. The parameters for improved ThiC activity and turnover described here will facilitate kinetic and mechanistic analyses of ThiC.

Numerous radical SAM enzymes have been identified by bioinformatics analysis, and those that have been characterized carry out diverse reactions within metabolism including nucleic acid modification and repair, and synthesis.
of cofactors and antibiotics. Enzymes in the radical SAM superfamily can be divided into three classes (11-13). The first class uses SAM as a catalytic cofactor and includes spore-photoproduction lyase (SPL) and lysine 2,3-aminomutase (LAM) (14,15). The second class is made up of glycol-radical activating enzymes, which catalyze radical formation on glycines in other enzymes. This class includes pyruvate formate-lyase activating enzyme (PFL-AE) and ribonucleotide reductase activating enzyme (RNR-AE) (16,17). Enzymes in the third class use SAM as a substrate. The majority of radical SAM enzymes characterized to date fall into this class, including lipoil synthase (LipA), tyrosine lyase (ThiH), and biotin synthase (BioB) (18-20).

According to the literature, ThiC uses SAM as an oxidizing co-substrate (1:1 stoichiometry) (4), making it a member of the third class described above. The activities of BioB, ThiH and LipA are inhibited by SAM cleavage products 5'-deoxyadenosine (5'-DOA) and Met (21,22), while other enzymes in this class (including the maturase from Klebsiella pneumonia AtsB and butirosin biosynthetic enzyme BtrN) are not product inhibited (23,24). S-methyl-5'-thioadenosine nucleosidase (MTAN; E.C. 3.2.2.9, 3.2.2.16) breaks down 5'-DOA to adenine and 5'-deoxyribose (25) and can improve activity when added to the assay mix of enzymes that are inhibited by 5'-DOA (21,22).

This study was motivated by our interest in the complex metabolic context of the ThiC reaction in Salmonella enterica. In this organism, the conversion of AIR to HMP-P was decreased by perturbations in other metabolic processes, including the biosynthetic pathways for purines, Met, iron-sulfur clusters and coenzyme A (CoA) (26-29). We sought to improve the in vitro assay for ThiC activity to allow us to obtain kinetic parameters that could help us rationalize the diverse metabolic connections identified in vivo. Here, we report assay conditions for the in vitro ThiC reaction that resulted in multiple turnovers and allowed the first kinetic measurements of this enzyme activity.

**EXPERIMENTAL PROCEDURES**

**Media and chemicals**—Difco Luria Bertani (LB; 20 g/L) medium was used for routine Escherichia coli growth. For protein overexpression, Superbroth (SB; tryptone (32 g/L), yeast extract (20 g/L), NaCl (5 g/L) with NaOH (0.05 N)) was used. Ampicillin and kanamycin were added to the medium as needed at 150 mg/L and 50 mg/L, respectively. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich, St Louis, MO.

**Protein purification and ThiC reconstitution**—Proteins flavoprotein reductase (Fpr; E.C. 1.18.1.2), flavodoxin A (FldA), and TdPurE were expressed and purified as previously described (26,30). TdPurE is Treponema denticola AIR carboxylase (E.C. 5.4.99.18), and was produced from pJK376, a gift from J. Kappock. All ThiC purifications and manipulations were carried out in an anoxic glovebox (Coy Laboratories, Grass Lake, MI) maintained at < 2 ppm O₂. S. enterica His₅-ThiC was produced from vector pET-28b(+) in a strain overexpressing Azotobacter vinelandii [Fe-S] cluster-loading genes from plasmid pDB1282 (31). ThiC was purified as described (26), except that the [4Fe-4S] cluster was reconstituted in vitro prior to freezing the protein at -80°C. After purification, ThiC concentration was determined by Pierce 660 assay (Thermo Scientific, Rockford, IL) using bovine serum albumin (BSA) as the standard.

ThiC protein was reduced by adding a 50% excess of dithiothreitol (DTT) in a vial that was then sealed and incubated on ice in the glovebox overnight. A fresh stock solution of FeNH₄SO₄ (400 mM) was added in four aliquots to be 8-fold in excess of ThiC and the vial was incubated at room temperature for 5 min. A fresh stock solution of Na₂S (400 mM) was then added in four aliquots to reach an 8-fold excess over ThiC. Reduced ThiC was incubated for 1 hr before desalting into Freezing Buffer (50 mM N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid sodium-potassium salt, [(2-hydroxy-1,1-bis(hydroxymethyl)ethylamino)-1-propanesulfonic (TAPS) pH 8.0, 0.2 M NaSO₄, 1.6 M glycerol) by a PD-10 Sephadex G-25 column (GE Healthcare Life Sciences, Piscataway, NJ). The desalted protein was concentrated in an Amicon 10,000 MWCO centrifugal filter unit (Millipore, Billerica, MA) at 2,400 x g in sealed centrifuge tubes outside of the glovebox. The protein concentration after reconstitution was 0.27 ± 0.03 mM as determined by Bradford assay using
purified ThiC with concentration determined by amino acid analysis as a standard.

Iron content determination—The iron content of the ThiC protein was determined by a colorimetric assay using 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine disodium salt trihydrate (Ferene) adapted from Kennedy et al. (32). All reagents were prepared in double distilled water and in new glassware or plasticware to prevent iron contamination. Twenty-five μL of ThiC sample dilutions and iron standard solutions (Sigma) were mixed with 25 μL HCl (0.12 N) in 1.5-ml microcentrifuge tubes and shaken gently. After incubation at 80°C for 10 min, reagents were added to each tube sequentially with vortexing after each addition: 125 μL ammonium acetate (0.96 M); 25 μL ascorbic acid (0.2 M); 25 μL sodium dodecyl sulfate (87 mM); 25 μL Ferene (30 mM). The samples were then centrifuged for 5 min at 9,000 x g, and the supernatant analyzed for absorbance at 593 nm using a SpectraMax plate spectrophotometer (Molecular Devices, Sunnyvale, CA) since Ferene absorbs at 593 nm when complexed to Fe²⁺. Iron content was 4.2 ± 0.7 mol Fe/mol ThiC in the preparation used in the studies described herein.

Synthesis of CAIR, CAIRs, AIR and AIRs—4-carboxyamidinomidazole ribotide (CAIR) and AIR were synthesized as described (26,30,33). The molar extinction coefficients ε₂₅₀=10,980 M⁻¹ cm⁻¹ and ε₂₅₀=3,270 M⁻¹ cm⁻¹ (34) were used to determine their respective concentrations. 4-carboxyamidinomidazole riboside (CAIRs) and 5-amidinomidazole riboside (AIRs) were generated from stocks of CAIR and AIR (10-15 mM) treated with rAPid alkaline phosphatase (Roche, Mannheim, Germany) at 37° for 15 min. The alkaline phosphatase was then heat inactivated by incubating at 80° for 3 min and cleared by centrifugation at 21,100 x g for 1 min. The supernatant was transferred to a new tube and degassed for 10 min before transfer to the glovebox.

Purification of SAM from a pharmaceutical source—Commercial sources of SAM have been found to be as little as 43% biologically active S,S-SAM (22). We previously found that, of the compounds absorbing at 259 nm, SAMe (NatureMade, Mission Hills, CA) was approximately 88% S,S-SAM by HPLC analysis (26). To further purify S,S-SAM, a SAMe pill was crushed, dissolved in ddH₂O and filtered through a 0.22 μm Spin-X filter (Corning). The concentration of adenine compounds was determined using the extinction coefficient ε₂₅₀=15,400 M⁻¹ cm⁻¹ (35) and the concentration was adjusted to 100 mM in ddH₂O. Three μl injections of the SAMe solution were separated by RP-HPLC with a LC-20AT delivery system (Shimadzu, Kyoto, Japan) equipped with a 250 x 4.6 mm Luna C18 (2), 5 micron chromatography resolution column (Phenomenex, Torrance, CA). The column was equilibrated with 90% Mobile Phase A (13 mM trifluoroacetic acid (TFA)), and 10% Mobile Phase B (methanol). The separation used a flow rate of 1 mL min⁻¹, with 90% A, 10% B, for 10 min followed by a linear gradient to 50% B over 20 min. Components eluted from the column were monitored with a SPD-M20A photodiode array detector (Shimadzu; wavelengths 190-350 nm), with data extracted at 259 nm. The 3.00-3.85 min fraction was collected using the FRC-10A fraction collector (Shimadzu, Kyoto, Japan), outfitted with a Styrofoam box filled with dry ice so the purified SAM was immediately frozen as it was collected in a 50-mL conical tube. The purified SAM was lyophilized and resuspended in ddH₂O sequentially three times to remove residual TFA. The purified SAM powder was resuspended in ddH₂O (~22 mM) and samples were frozen at -20° until use. HPLC analysis determined the purified SAM was 99% pure.

ThiC activity assays—Fpr, FldA, MTAN, SAM and AIR were degassed with nitrogen for 10 min in 1.5-mL microcentrifuge tubes sealed with rubber stoppers prior to being placed in the glovebox. Concentrations of AIR and SAM were determined with a Nanodrop spectrophotometer (Thermo Scientific) using the extinction coefficients listed above.

All components were resuspended in anoxic Reaction Buffer (50 mM TAPS, pH 8.0). Each assay included ThiC (0.55 nmol monomer, 11 μM), MTAN (as indicated, 0.1 nmol), Fpr (0.5 nmol) and FldA (1 nmol). Under these conditions, HMP production was linear with respect to ThiC concentration, and MTAN, Fpr and FldA were not rate limiting. Reduced nicotinamide adenine dinucleotide phosphate (NADPH, 0.8 mM) was added in excess and the reaction mix incubated for 10 min at room temperature before adding the substrate of interest. Substrates SAM (25-150 μM),
and AIR (25-150 μM) were added to a final volume of 50 μL. The reactions were incubated at 37°C in the anaerobic chamber for the specified time, stopped by heat treatment (65°C for 3 min) and frozen at -20°C if they were not analyzed immediately. When included, inhibitors were pre-incubated with the ThiC reaction mixture for 10 min before the relevant substrates were added. Homocysteine, AICA, Met, adenosine and imidazole were brought into the glovebox as powders and resuspended in anoxic Reaction Buffer; adenosine was heated at 65°C for 5 min to dissolve. All other potential inhibitors were made in Reaction Buffer, adjusted to pH 6-9, and degassed for 10 min prior to entering the glovebox. In assays where we titrated specific inhibitors, the concentration of the inhibitor was determined after degassing using the relevant extinction coefficient. MTAN was not used in assays addressing inhibition.

HMP-P was dephosphorylated to HMP by alkaline phosphatase and quantified as described (26). In addition, samples were filtered through a 10,000-50,000 kDa cellulose membrane with an Amicon centrifugal filter (Millipore, Billerica, MA) to remove proteins prior to transferring the samples to autosampler vials (Macherey-Nagel, Duren, Germany).

Kinetic data analysis—Graphs were prepared and data were analyzed using least squares analysis in Prism v. 6.0b (Graphpad Software Inc., La Jolla, CA). Kinetic constants are reported with the standard error of the fit, unless otherwise noted. For time course experiments, the data were fitted to a first-order kinetic equation, Equation 1, where [HMP] was the observed HMP produced (μM); [HMP]max was the predicted maximum HMP produced (μM); k was the observed first-order rate constant; and t was time in min.

\[
[HMP] = [HMP]_{\text{max}}(1 - e^{-kt})
\]  

(1)

The initial turnover number, \(k_{\text{cat}}^0\), was determined by Equation 2, based on the methods of Challand et al. (36).

\[
k_{\text{cat}}^0 = \frac{k[HMP]_{\text{max}}}{[\text{ThiC monomer}]}
\]  

(2)

To determine the kinetics of ThiC inhibition, the initial velocity (\(v\); nmol HMP/nmol ThiC/min) was estimated from reactions stopped after 20 min incubation at 37°C. The \(K_m\) was determined from data titrating SAM (20-150 μM) and omitting MTAN. The data were fit to Equation 3.

\[
v = \frac{V_{\text{max}}[S]}{K_m+[S]}
\]  

(3)

Data were first diagnosed as competitive, uncompetitive or noncompetitive inhibition by their appearance when graphed as double reciprocal Lineweaver-Burk plots and fit by linear regression. The data were then analyzed according to the appropriate equation. For competitive inhibition, Equation 4 was used, where \(v\) is the velocity in nmol HMP/nmol ThiC/min; \(V_{\text{max}}\) is the maximum velocity observed; \(K_{\text{mObs}}\) is determined by the equation \(K_{\text{mObs}} = K_m(1 + [I]/K_i);\) and [S] is the concentration of substrate provided.

\[
\frac{1}{v} = \frac{K_{\text{mObs}}+[S]}{V_{\text{max}}[S]}
\]  

(4)

For cooperative competitive inhibition by two different nonexclusive inhibitors, the data were fit to Equation 5 (37), where \(v\) is the velocity in nmol HMP/nmol ThiC/min; \(V_{\text{max}}\) is the maximum velocity; [S] is the concentration of substrate; \(K_x\) is the Michaelis-Menten constant for the substrate; [I] is the concentration of one inhibitor, and \(K_i\) is its inhibition constant; and [X] is the concentration of the second inhibitor, and \(K_x\) is its inhibition constant; and \(\alpha\) is the cooperativity factor.

\[
\frac{v}{V_{\text{max}}} = \frac{[S]}{K_x} \frac{1 + [I]/K_i}{1 + [X]/K_x} \frac{1 + [I]/K_i}{1 + [X]/K_x} \alpha K_i K_x
\]  

(5)

For uncompetitive inhibition, Equation 6 was used, where \(v\) is the velocity in nmol HMP/nmol ThiC/min; \(V_{\text{maxApp}}\) is the apparent maximum velocity; \(K_{\text{mApp}}\) is the apparent \(K_m\); and the \(K_i^*\) inhibition constant is determined by the equations \(V_{\text{maxApp}} = V_{\text{max}}/(1+([I]/(K_i^*)))\) and \(K_{\text{mApp}} = K_m/(1 + ([I]/(K_i^*))).\) The \(V_{\text{max}}\) for this dataset was determined by fitting the data for [Ado] = 0 μM to Equation 3.
RESULTS and DISCUSSION

**ThiC is a multiple turnover enzyme**—ThiC activity assays described elsewhere required high protein concentration and/or long incubations to quantify HMP-P production (1-4,26). These conditions prevented mechanistic and kinetic analysis of ThiC. Changes were made to the assay protocol for ThiC to increase HMP production. The [4Fe-4S] cluster in ThiC was reconstituted in vitro and pure sources of substrates AIR and SAM (99% pure) were used in the assay. With these modifications, ThiC produced 3.1 ± 0.1 nmol HMP/nmol ThiC monomer in 2 hr, confirming that multiple turnovers were possible in vitro (Figure 2). Under these conditions, steady state turnover continued for 25 min. Data from technical duplicates were fit to the first-order kinetic equation (Equation 1) with a goodness of fit $R^2$ of $> 0.95$. These results were then used in Equation 2 and yielded the following turnover number, representing the average and standard error of the constants determined by two independent experiments: $k_{cat}^0 = 0.074 ± 0.014 \text{ min}^{-1}$.

The production of HMP was significantly enhanced by the addition of MTAN. When 0.1 nmol MTAN was included in the reaction mix, ThiC produced 5.2 ± 0.1 nmol HMP/nmol ThiC and steady state turnover continued for 1 hr. The kinetic analysis of these data yielded the turnover number $k_{cat}^0 = 0.14 ± 0.03 \text{ min}^{-1}$. This value for $k_{cat}^0$ is within the range reported for other Radical SAM enzymes in this class (22-24,36,38).

**SAM-related metabolites inhibit the ThiC in vitro reaction**—The finding that MTAN increased the reaction rate by approximately two-fold suggested ThiC was inhibited by its product 5'-DOA. This conclusion was verified and extended by screening a number of potentially relevant metabolites for an effect on ThiC activity. Potential inhibitors tested included: SAM-related metabolites, purines related to the substrate AIR, aminomimidazole carboxamide ribotide (AICAR)-related metabolites and CoA metabolites. The latter two represented metabolic pathways shown to impact the AIR to HMP-P conversion in vivo (28,29).

$\frac{1}{v} = \frac{K_{m,App} + [S]}{v_{max,App} [S]}$

(6)

Under the conditions tested, we saw no inhibition by purine biosynthetic intermediates related to AIR, including imidazole and the AIR riboside. These data support the conclusion that the in vivo findings reflect indirect metabolic effects of AICAR and CoA on the ThiC reaction. In contrast, several SAM-related metabolites inhibited ThiC, specifically: 5'-DOA, Met, homocysteine (Hcy), adenosine (Ado), S-adenosyl/homocysteine (SAH), and S-methyl-5'-thioadenosine (MTA) (Figure 3). Of these metabolites, 5'-DOA, Met, Hcy, and SAH are known inhibitors of radical SAM enzymes (reviewed in (13)). The data also showed that 5'-DOA acted additively with either Met or Hcy to further inhibit ThiC activity.

**S-adenosyl/homocysteine inhibits ThiC competitively with SAM**—SAH has been reported to inhibit representatives of all three classes of radical SAM enzymes: LAM, RNR-AE, BioB and the nitrogenase cofactor biosynthetic enzyme NifB (22,39-41). The mechanism of SAH inhibition of ThiC was investigated by adding SAH at different concentrations (0, 10, 25 and 50 µM) to reaction mixtures containing several concentrations of SAM (25-150 µM). The Lineweaver-Burk plot of these data showed that SAH inhibited ThiC competitively with SAM (Figure 4). The $K_m$ of ThiC for SAM was determined by fitting data to Equation 3 from duplicate reactions of a titration of SAM (20-150 µM) carried out without MTAN. The data were fit with a global $R^2$ value of 0.89 and the $K_m$ was $17 ± 3 \mu M$. Based the diagnosis of competitive inhibition, the data were fit to Equation 4 using the above $K_m$ with global $R^2$ value of 0.85 and generated the kinetic constant $K_{SAH}^{5'} = 5.6 ± 1.1 \mu M$.

In the cell, SAH is produced by SAM methyltransferases and hydrolyzed by MTAN (42). SAH is present at approximately 1 µM in wild-type E. coli and 50 µM in a mutant strain without MTAN (43). Together, these data suggest SAH could have a physiologically relevant role in regulating ThiC activity under conditions where MTAN activity is reduced.

5'-Deoxyadenosine and methionine cooperatively inhibit ThiC—5'-DOA and Met were found to cooperatively inhibit BioB (22), and data from our inhibitor screen indicated that they also cooperatively inhibited ThiC. The reduction
ThiC turnover and inhibition by SAM metabolites

in activity by the addition of 5'-DOA and Met together (12% of activity with no inhibitor) was slightly greater than expected for linear combination of the inhibition caused by 5'-DOA (31%) or Met (55%) when either was the sole addition. To investigate the kinetics of this inhibition, several concentrations of 5'-DOA (0-500 μM) and Met (0-1000 μM) were added to ThiC reactions with AIR and SAM fixed at 100 μM (Figure 5). Dixon replots of 1/v vs. [5'-DOA] or [Met] intersected, confirming that 5'-DOA and Met were non-mutually exclusive (37). 5'-DOA and Met were assumed to inhibit competitively with respect to SAM. The least squares analysis was constrained to [S] = 100 μM and $K_m = 17$ μM and the data fit Equation 5 with a global $R^2$ value of 0.94 and yielded: $K_i^{5'-DOA} = 12 \pm 2$ μM; $K_i^{Met} = 82 \pm 13$ μM and $\alpha = 0.4 \pm 0.1$.

Under normal metabolic conditions, product inhibition would be expected to be minimal; Met concentrations are estimated at 150-300 μM (43,44) and MTAN is present to rapidly hydrolyze low levels of 5'-DOA produced. However, these constants suggest that product inhibition could be significant in *in vitro* assays, including those reported here. For example, after 2 hr incubation product accumulation coupled with substrate depletion would cause ThiC to be 60% or 35% maximal activity with or without MTAN, respectively. These findings suggest long incubation times will not allow accurate kinetic measurements of ThiC.

Adenosine displays uncompetitive inhibition with SAM—If adenosine bound the site occupied by the adenosine moiety of SAM, adenosine should also inhibit ThiC competitively with respect to SAM. Adenosine was added at several concentrations (0, 100, 250 and 400 μM) to reactions containing several SAM concentrations (25-150 μM). Unexpectedly, the data with and without adenosine resulted in parallel lines in the Lineweaver-Burk plot (Figure 6A), suggesting that adenosine was uncompetitive with SAM and bound the ThiC-SAM complex. To determine $V_{max}$, the [Ado] = 0 μM data were fit to Equation 3 with $R^2$ value of 0.92 to yield $V_{max} = 0.1128 \pm 0.0034$ nmol HMP/nmol ThiC/min. The full dataset was fit to Equation 6, constraining the $K_m = 17$ μM and $V_{max} = 0.1128$ nmol HMP/nmol ThiC/min. The data fit Equation 6 with a global $R^2$ value of 0.91, and produced the kinetic constant $K_i^{Ado} = 99 \pm 3$ μM. However, the uncertainty in the inhibition constant is likely considerably higher; we found $K_i^{Ado}$ values of 55-140 μM were consistent with the data. Replots of the data from the reciprocal Lineweaver-Burk plot were also linear, confirming the diagnosis of uncompetitive inhibition (37). Experiments addressing adenosine inhibition with respect to AIR showed that adenosine is not competitive with AIR, which is consistent with the fact that AMP does not inhibit. The data did not distinguish between uncompetitive and noncompetitive inhibition (data not shown).

The adenosine concentration in *E. coli* was estimated at 0.13 μM (44), suggesting adenosine inhibition is not physiologically relevant. However, direct inhibition of ThiC may be significant under conditions of increased adenosine levels, such as with AICAR accumulation (45) or when adenosine is present in the growth medium (46).

Conclusions—ThiC is the HMP-P synthase required for thiamine biosynthesis in bacteria and plants and is a member of the radical SAM superfamily of enzymes. Of numerous radical SAM enzymes predicted by bioinformatic analyses, relatively few have been characterized, and fewer still have been shown to turnover catalytically in *vitro* (10,38,47). The data presented herein demonstrate that once product inhibition is relieved, ThiC undergoes steady-state turnover for up to one hour.

To our knowledge, there are no other reports of radical SAM enzymes inhibited by adenosine or MTA, suggesting this may be a unique property of ThiC. Although not many enzymes have been tested, BioB was not inhibited by adenosine or MTA (22) and MTA was reported to have no effect on LAM activity (39). Thus, ThiC has a distinct inhibitor profile, in addition to its variant cysteine motif and proposed novel catalytic mechanism. The characterization of ThiC activity presented here, in particular achieving catalytic turnover in *vitro*, will contribute to future mechanistic studies of ThiC and furthering our understanding of the radical SAM enzyme superfamily.
REFERENCES

1. Martinez-Gomez, N. C., and Downs, D. M. (2008) ThiC is an [Fe-S] cluster protein that requires AdoMet to generate the 4-amino-5-hydroxymethyl-2-methylpyrimidine moiety in thiamin synthesis. *Biochemistry* **47**, 9054-9056

2. Martinez-Gomez, N. C., Poyner, R. R., Mansoorabadi, S. O., Reed, G. H., and Downs, D. M. (2009) Reaction of AdoMet with ThiC generates a backbone free radical. *Biochemistry* **48**, 217-219

3. Chatterjee, A., Li, Y., Zhang, Y., Grove, T. L., Lee, M., Krebs, C., Booker, S. J., Begley, T. P., and Ealick, S. E. (2008) Reconstitution of ThiC in thiamine pyrimidine biosynthesis expands the radical SAM superfamily. *Nat. Chem. Biol.* **4**, 758-765

4. Chatterjee, A., Hazra, A. B., Abdelwahed, S., Hilmy, D. G., and Begley, T. P. (2010) A "radical dance" in thiamin biosynthesis: mechanistic analysis of the bacterial hydroxymethylpyrimidine phosphate synthase. *Angew. Chem. Int. Ed. Eng.* **49**, 8653-8656

5. Begley, T. P., Downs, D. M., Ealick, S. E., McLafferty, F. W., Van Loon, A. P., Taylor, S., Campobasso, N., Chiu, H. J., Kinsland, C., Reddick, J. J., and Xi, J. (1999) Thiamin biosynthesis in prokaryotes. *Arch. Microbiol.* **171**, 293-300

6. Begley, T. P., Chatterjee, A., Hanes, J. W., Hazra, A., and Ealick, S. E. (2008) Cofactor biosynthesis--still yielding fascinating new biological chemistry. *Curr. Opin. Chem. Biol.* **12**, 118-125

7. Estramareix, B., and David, S. (1990) Conversion of 5-aminoimidazole ribotide to the pyrimidine of thiamin in enterobacteria: study of the pathway with specifically labeled samples of riboside. *Biochim. Biophys. Acta* **1035**, 154-160

8. Estramareix, B., and Lesieur, M. (1969) Biosynthesis of the pyrimidine portion of thiamine: source of carbons 2 and 4 in *Salmonella typhimurium*. *Biochim. Biophys. Acta* **192**, 375-377

9. Estramareix, B., and Therisod, M. (1984) Biosynthesis of thiamine: 5-aminoimidazole ribotide as the precursor of all the carbon-atoms of the pyrimidine moiety. *J. Am. Chem. Soc.* **106**, 3857-3860

10. Sofia, H. J., Chen, G., Hetzler, B. G., Reyes-Spindola, J. F., and Miller, N. E. (2001) Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res.* **29**, 1097-1106

11. Frey, P. A., and Booker, S. J. (1999) Radical intermediates in the reaction of lysine 2,3-Aminomutase. in *Advances in Free Radical Chemistry* (Zard, S. Z. ed.), JAI Press Inc., Stamford, CT. pp 1-43

12. Booker, S. J. (2009) Anaerobic functionalization of unactivated C-H bonds. *Curr. Opin. Chem. Biol.* **13**, 58-73

13. Hiscox, M. J., Driesener, R. C., and Roach, P. L. (2012) Enzyme catalyzed formation of radicals from S-adenosylmethionine and inhibition of enzyme activity by the cleavage products. *Biochim. Biophys. Acta* **1824**, 1165-1177

14. Cheek, J., and Broderick, J. B. (2002) Direct H atom abstraction from spore photoprod C-6 initiates DNA repair in the reaction catalyzed by spore photoprod lyase: evidence for a reversibly generated adenosyl radical intermediate. *J. Am. Chem. Soc.* **124**, 2860-2861
15. Moss, M. L., and Frey, P. A. (1990) Activation of lysine 2,3-aminomutase by S-adenosylmethionine. J. Biol. Chem. 265, 18112-18115
16. Wagner, A. F., Frey, M., Neugebauer, F. A., Schafer, W., and Knappe, J. (1992) The free radical in pyruvate formate-lyase is located on glycine-734. Proc. Natl. Acad. Sci. U.S.A. 89, 996-1000
17. Tamarit, J., Mulliez, E., Meier, C., Trautwein, A., and Fontecave, M. (1999) The anaerobic ribonucleotide reductase from Escherichia coli. The small protein is an activating enzyme containing a [4Fe-4S](2+) center. J. Biol. Chem. 274, 31291-31296
18. Miller, J. R., Busby, R. W., Jordan, S. W., Checek, J., Henshaw, T. F., Ashley, G. W., Broderick, J. B., Cronan, J. E., Jr., and Marletta, M. A. (2000) Escherichia coli LipA is a lipoyl synthase: in vitro biosynthesis of lipoylated pyruvate dehydrogenase complex from octanoyl-acyl carrier protein. Biochemistry 39, 15166-15178
19. Kriek, M., Martins, F., Leonardi, R., Fairhurst, S. A., Lowe, D. J., and Roach, P. L. (2007) Thiazole synthase from Escherichia coli: an investigation of the substrates and purified proteins required for activity in vitro. J. Biol. Chem. 282, 17413-17423
20. Ollagnier-de-Choudens, S., Mulliez, E., and Fontecave, M. (2002) The PLP-dependent biotin synthase from Escherichia coli: mechanistic studies. FEBS Lett. 532, 465-468
21. Challand, M. R., Ziegert, T., Douglas, P., Wood, R. J., Kriek, M., Shaw, N. M., and Roach, P. L. (2009) Product inhibition in the radical S-adenosylmethionine family. FEBS Lett. 583, 1358-1362
22. Farrar, C. E., Siu, K. K., Howell, P. L., and Jarrett, J. T. (2010) Biotin synthase exhibits burst kinetics and multiple turnovers in the absence of inhibition by products and product-related biomolecules. Biochemistry 49, 9985-9996
23. Grove, T. L., Lee, K. H., St Clair, J., Krebs, C., and Booker, S. J. (2008) In vitro characterization of AtsB, a radical SAM formylglycine-generating enzyme that contains three [4Fe-4S] clusters. Biochemistry 47, 7523-7538
24. Yokoyama, K., Numakura, M., Kudo, F., Ohmori, D., and Eguchi, T. (2007) Characterization and mechanistic study of a radical SAM dehydrogenase in the biosynthesis of butirosin. J. Am. Chem. Soc. 129, 15147-15155
25. Choi-Rhee, E., and Cronan, J. E. (2005) A nucleosidase required for in vivo function of the S-adenosyl-L-methionine radical enzyme, biotin synthase. Chem. Biol. 12, 589-593
26. Palmer, L. D., Dougherty, M. J., and Downs, D. M. (2012) Analysis of ThiC variants in the context of the metabolic network of Salmonella enterica. J. Bacteriol. 194, 6088-6095
27. Dougherty, M. J., and Downs, D. M. (2006) A connection between iron-sulfur cluster metabolism and the biosynthesis of 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate in Salmonella enterica. Microbiology 152, 2345-2353
28. Allen, S., Zilles, J. L., and Downs, D. M. (2002) Metabolic flux in both the purine mononucleotide and histidine biosynthetic pathways can influence synthesis of the hydroxymethyl pyrimidine moiety of thiamine in Salmonella enterica. J. Bacteriol. 184, 6130-6137
29. Frodyma, M., Rubio, A., and Downs, D. M. (2000) Reduced flux through the purine biosynthetic pathway results in an increased requirement for coenzyme A in thiamine synthesis in Salmonella enterica serovar typhimurium. J. Bacteriol. 182, 236-240
30. Tranchimand, S., Starks, C. M., Mathews, II, Hocking, S. C., and Kappock, T. J. (2011) Treponema denticola PurE is a bacterial AIR carboxylase. Biochemistry 50, 4623-4637
31. Cicchillo, R. M., Lee, K. H., Baleanu-Gogonea, C., Nesbitt, N. M., Krebs, C., and Booker, S. J. (2004) *Escherichia coli* lipoamide synthase binds two distinct [4Fe-4S] clusters per polypeptide. *Biochemistry* **43**, 11770-11781

32. Kennedy, M. C., Kent, T. A., Emptage, M., Merkle, H., Beinert, H., and Munck, E. (1984) Evidence for the formation of a linear [3Fe-4S] cluster in partially unfolded aconitase. *J. Biol. Chem.* **259**, 14463-14471

33. Mehl, R. A., and Begley, T. P. (2002) Synthesis of P-32-labeled intermediates on the purine biosynthetic pathway. *J. Labelled Comp. Radiopharm.* **45**, 1097-1102

34. Meyer, E., Leonard, N. J., Bhat, B., Stubbe, J., and Smith, J. M. (1992) Purification and characterization of the purE, purK, and purC gene-products - identification of a previously unrecognized energy requirement in the purine biosynthetic-pathway. *Biochemistry* **31**, 5022-5032

35. Bock, R. M., Ling, N. S., Morell, S. A., and Lipton, S. H. (1956) Ultraviolet absorption spectra of adenosine-5'-triphosphate and related 5'-ribonucleotides. *Arch. Biochem. Biophys.* **62**, 253-264

36. Challand, M. R., Martins, F. T., and Roach, P. L. (2010) Catalytic activity of the anaerobic tyrosine lyase required for thiamine biosynthesis in *Escherichia coli*. *J. Biol. Chem.* **285**, 5240-5248

37. Segel, I. H. (1975) *Enzyme Kinetics*, John Wiley & Sons, Inc., New York

38. Roach, P. L. (2011) Radicals from S-adenosylmethionine and their application to biosynthesis. *Curr. Opin. Chem. Biol.* **15**, 267-275

39. Chirpich, T. P., Zappia, V., Costilow, R. N., and Barker, H. A. (1970) Lysine 2,3-aminomutase. Purification and properties of a pyridoxal phosphate and S-adenosylmethionine-activated enzyme. *J. Biol. Chem.* **245**, 1778-1789

40. Harder, J., Eliasson, R., Pontis, E., Ballinger, M. D., and Reichard, P. (1992) Activation of the anaerobic ribonucleotide reductase from *Escherichia coli* by S-adenosylmethionine. *J. Biol. Chem.* **267**, 25548-25552

41. Curatti, L., Ludden, P. W., and Rubio, L. M. (2006) NifB-dependent *in vitro* synthesis of the iron-molybdenum cofactor of nitrogenase. *Proc. Natl. Acad. Sci. U.S.A* **103**, 5297-5301

42. Walker, R. D., and Duerre, J. A. (1975) S-adenosylhomocysteine metabolism in various species. *Can. J. Biochem.* **53**, 312-319

43. Halliday, N. M., Hardie, K. R., Williams, P., Winzer, K., and Barrett, D. A. (2010) Quantitative liquid chromatography-tandem mass spectrometry profiling of activated methyl cycle metabolites involved in LuxS-dependent quorum sensing in *Escherichia coli*. *Anal. Biochem.* **403**, 20-29

44. Bennett, B. D., Kimball, E. H., Gao, M., Osterhout, R., Van Dien, S. J., and Rabinowitz, J. D. (2009) Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat. Chem. Biol.* **5**, 593-599

45. Kuramitsu, H. K., Udaka, S., and Moyed, H. S. (1964) Induction of inosine 5'-phosphate dehydrogenase and xanthosine 5'-phosphate aminase by ribosyl-4-amino-5-imidazolecarboxamide in purine-requiring mutants of *Escherichia coli*. *B. J. Biol. Chem.* **239**, 3425-3430

46. Moyed, H. S. (1964) Inhibition of the biosynthesis of the pyrimidine portion of thiamine by adenosine. *J. Bacteriol.* **88**, 1024-1029
47. Shisler, K. A., and Broderick, J. B. (2012) Emerging themes in radical SAM chemistry. *Curr. Opin. Struct. Biol.* **22**, 701-710
Acknowledgments—We thank Jorge C. Escalante-Semerena for critical reading of the manuscript and George H. Reed and Michael G. Thomas for helpful discussion. We thank T. Joseph Kappock for pJK376 expressing Td/PurE, Jannell V. Bazurto for providing MTAN, and Mackenzie J. Parker and JoAnne Stubbe for authentic HMP used for quantification.

FOOTNOTES
*The National Institutes of Health grant GM47296 to DMD supported this work. LDP was supported by NSF through Graduate Research Fellowship grant DGE-0718123.

1To whom correspondence should be addressed: Diana M. Downs, Department of Microbiology, University of Georgia, 527 Biological Sciences Building, 120 Cedar St, Athens, GA, USA, Tel.: (706) 542-7953; E-mail: dmdowns@uga.edu

FIGURE LEGENDS

FIGURE 1. ThiC reaction. Abbreviations: 5-aminoimidazole ribotide, AIR; 4-amino-hydroxymethyl-2-methylpyrimidine phosphate, HMP-P.

FIGURE 2. ThiC undergoes steady-state turnover. ThiC (0.55 nmol monomer) was incubated with flavoprotein reductase (0.5 nmol), flavodoxin A (1 nmol), NADPH (0.8 mM), SAM (100 µM), and AIR (100 µM) and MTAN as indicated (0.1 nmol) at 37°C. Each data point represents the average and standard deviation of two replicates from a single experiment. The data were fit to a first-order rate equation, and the 95% confidence intervals of the regression analysis are represented by dotted lines.

FIGURE 3. Metabolite inhibitors of ThiC activity. ThiC (0.55 nmol monomer) was pre-incubated with flavoprotein reductase (0.5 nmol), flavodoxin A (1 nmol), NADPH (0.8 mM) and potential inhibitor (0.5 mM) for 10 min at room temperature. Then SAM (100 µM), and AIR (100 µM) were added to initiate the reactions, which were incubated at 37°C for 30 min. Data represent the average and standard deviation of two replicates. An asterisk indicates that the average is significantly different than the average with no inhibitor, as determined by an unpaired t-test (p<0.05). Abbreviations: 4-amino-5-hydroxymethyl-2-methylpyrimidine, HMP; 5'-deoxyadenosine, 5'-DOA; methionine, Met; homocysteine, HCy; 2'-deoxyadenosine, 2'-DOA; adenosine, Ado; adenine, Ade; methionine, Met; S-adenosylhomocysteine, SAH; S-methyl-5'-thiodenosine, MTA; 5-aminoimidazole riboside, AIRs; aminomimidazole carboxamid ribotidate, AICAR; aminomimidazole carboxamide riboside, AICARs; aminomimidazole carboxamide, AICA; 5-Amino-4-imidazolecarboxylic acid ribotide, CAIR; 5-Amino-4-imidazolecarboxylic acid riboside, CAIRs.

Figure 4. SAH inhibits ThiC competitively with respect to SAM. ThiC (0.55 nmol monomer) was pre-incubated with flavoprotein reductase (0.5 nmol), flavodoxin A (1 nmol), NADPH (0.8 mM), AIR (100 µM) and adenosine (0, 100, 250 or 400 µM) for 10 min at room temperature. Then SAM (25-150 µM) was added to initiate the reactions, which were incubated at 37°C for 20 min. The data were fit to Equation 4 by non-linear regression, constraining $K_m = 17$ µM. Abbreviations: S-adenosylhomocysteine, SAH.

Figure 5. Cooperative inhibition by 5'-DOA and Met. ThiC (0.55 nmol monomer) was pre-incubated with flavoprotein reductase (0.4 nmol), flavodoxin A (1 nmol), NADPH (0.8 mM), 5'-DOA (0-500 µM) and Met (0-1000 µM) for 10 minutes at room temperature. Then SAM (100 µM) and AIR (100 µM) were added to initiate the reactions, which were incubated at 37°C for 20 minutes. The data were fit to Equation 5 by non-linear regression, constraining $K_m = 17$ µM and [SAM] = 100 µM. Abbreviations: methionine, Met; 5'-deoxyadenosine, 5'-DOA.
Figure 6. Adenosine is uncompetitive with SAM inhibiting ThiC. A. ThiC (0.55 nmol monomer) was pre-incubated with flavoprotein reductase (0.5 nmol), flavodoxin A (1 nmol), NADPH (0.8 mM), AIR (100 µM) and adenosine (0, 100, 250 or 400 µM) for 10 min at room temperature. Then SAM (25-150 µM) was added to initiate the reactions, which were incubated at 37°C for 20 min. The data were fit to Equation 6 by non-linear regression, constraining $K_m = 17$ µM and $V_{max} = 0.1128$ nmol HMP/nmol ThiC/min. Abbreviations: adenosine, Ado.
Figure 1.
Figure 2.
Figure 3.

ThiC turnover and inhibition by SAM metabolites

![Bar chart showing nmol HMP/nmol ThiC for various inhibitors and metabolites.](image-url)
Figure 4.

![Graph showing ThiC turnover and inhibition by SAM metabolites](image-url)
ThiC turnover and inhibition by SAM metabolites

Figure 5.
Figure 6.

\[ \frac{1}{\nu} \text{ (nmol ThiC \cdot min/nmol HMP)} \]

vs.

\[ \frac{1}{\text{SAM (\muM)}^{-1}} \]

Ado (\muM)

0 0.01 0.02 0.03 0.04

0 20 40 60 80 100 120 140 160
