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

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Background: ThiC is a radical *S*-adenosylmethionine (AdoMet) 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 AdoMet 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 AdoMet metabolites that inhibit ThiC activity.

ThiC (4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase; EC 4.1.99.17) is a radical *S*-adenosylmethionine (AdoMet) enzyme that uses a $[4Fe\text{-}4S]^{+}$ cluster to reductively cleave AdoMet to methionine and a 5′-deoxyadenosyl radical that initiates catalysis. In plants and bacteria, ThiC converts the purine intermediate 5-aminoimidazole ribotide to 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate, 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 AdoMet 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 AdoMet 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[2] synthase, EC 4.1.99.17) is a radical *S*-adenosylmethionine (AdoMet) enzyme that catalyzes the intramolecular rearrangement of 5-aminoimidazole ribotide (AIR) into HMP-P, carbon monoxide, and formate (Fig. 1) (1–4). HMP-P is condensed with 4-methyl-5-β-hydroxyethylthiazole phosphate to generate thiamine phosphate, which is further phosphorylated to the biologically active cofactor thiamine pyrophosphate (reviewed in Refs. 5, 6). Metabolically, AIR is at the branch point of purine and thiamine biosynthesis and has been known for decades to be the sole source of carbon for HMP-P (7–9). ThiC binds a $[4Fe\text{-}4S]^{2+}$ cluster with a CX_2CX_4C motif (1), a unique variation on the canonical radical AdoMet superfamily motif CX_3CX_2C (10). Once reduced, the $[4Fe\text{-}4S]^{+}$ cluster reductively cleaves AdoMet, producing methionine (Met) and a 5′-deoxyadenosyl radical that initiates catalysis. *In vitro* work by Chatterjee *et al.* (4) suggested that ThiC catalysis used two sequential hydrogen abstractions by the 5′-deoxyadenosyl radical, a mechanism that had not been reported.

Numerous radical AdoMet 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 AdoMet superfamily can be divided into three classes (11–13). The first class uses AdoMet as a catalytic cofactor and includes spore photoproduct lyase and lysine 2,3-aminomutase (14, 15). The second class is made up of glycyl radical-activating enzymes that catalyze radical formation on glycines in other enzymes. This class includes pyruvate formate-lyase activating enzyme and ribonucleotide reductase-activating enzyme (16, 17). Enzymes in the third class use AdoMet as a substrate. The majority of radical AdoMet enzymes characterized to date fall into this class, including lipoyl synthase, tyrosine lyase, and biotin synthase (BioB) (18–20).

According to the literature, ThiC uses AdoMet as an oxidizing cosubstrate (1:1 stoichiometry) (4), making it a member of the third class described above. The activities of BioB, tyrosine lyase, and lipoyl synthase are inhibited by AdoMet cleavage products 5′-deoxyadenosine (5′-DOA) and Met (21, 22), whereas other enzymes in this class (including the maturase from *Klebsiella pneumoniae* AtsB and butirosin biosynthetic enzyme BtrN) are not product-inhibited (23, 24). *S*-methyl-5′-

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[2] The abbreviations used are: HMP-P, 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate; AdoMet, *S*-adenosylmethionine; AIR, 5-aminoimidazole ribotide; BioB, biotin synthase; 5′-DOA, 5′-deoxyadenosine; MTAN, *S*-methyl-5′-thioadenosine nucleosidase; TAPS, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid; CAIR, 5-Amino-4-imidazolecarboxylic acid ribotide; HMP, 4-amino-5-hydroxymethyl-2-methylpyrimidine; AICAR, aminoimidazole carboxamide ribotide; Ado, adenosine; SAH, *S*-adenosylhomocysteine; MTA, *S*-methyl-5′-thioadenosine.

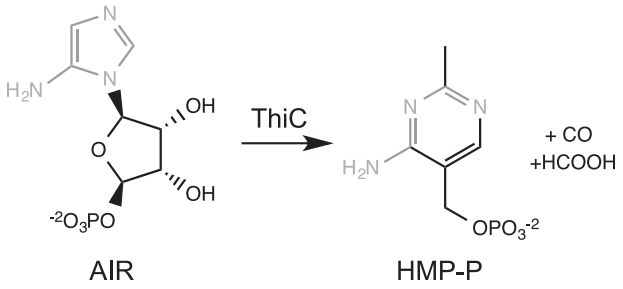

FIGURE 1. **ThiC reaction.**

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 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 (20 g/L) medium was used for routine *Escherichia coli* growth. For protein overexpression, Superbroth (tryptone (32 g/liter), yeast extract (20 g/liter), and NaCl (5 g/liter) with NaOH (0.05 N)) was used. Ampicillin and kanamycin were added to the medium as needed at 150 mg/liter and 50 mg/liter, respectively. Unless noted otherwise, 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 *Td*PurE were expressed and purified as described previously (26, 30). *Td*PurE 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 glove box (Coy Laboratories, Grass Lake, MI) maintained at < 2 ppm O_2. *S. enterica* His_6-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 BSA as the standard.

ThiC protein was reduced by adding a 50-fold excess of DTT in a vial that was then sealed and incubated on ice in the glove box overnight. A fresh stock solution of $FeNH_3SO_4$ (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 Na_2S (400 mM) was then added in four aliquots to reach an 8-fold excess over ThiC. Reduced ThiC was incubated for 1 h before desalting into freezing buffer (50 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid sodium-potassium salt, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}-1-propanesulfonic acid (TAPS) (pH 8.0), 0.2 M Na_2SO_4, 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 Da molecular weight cut off centrifugal filter unit (Millipore, Billerica, MA) at 2400 × *g* in sealed centrifuge tubes outside of the glove box. The protein concentration after reconstitution was 0.27 ± 0.03 mM, as determined by Bradford assay using purified ThiC with the 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 adapted from Kennedy *et al.* (32). All reagents were prepared in double-distilled water and in new glassware or plasticware to prevent iron contamination. 25 μl of ThiC sample dilutions and iron standard solutions (Sigma) were mixed with 25 μl of 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 of ammonium acetate (0.96 M), 25 μl of ascorbic acid (0.2 M), 25 μl of sodium dodecyl sulfate (87 mM), and 25 μl of 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine disodium salt trihydrate (30 mM). The samples were then centrifuged for 5 min at 9000 × *g*, and the supernatant was analyzed for absorbance at 593 nm using a SpectraMax plate spectrophotometer (Molecular Devices, Sunnyvale, CA) because 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine disodium salt trihydrate absorbs at 593 nm when complexed to Fe^{2+}. Iron content was 4.2 ± 0.7 mol iron/mol ThiC in the preparation used in the studies described herein.

Synthesis of CAIR, 4-Carboxyaminoimidazole Riboside, AIR, and Aminoimidazole Carboxamide Ribotide—CAIR and AIR were synthesized as described (26, 30, 33). The molar extinction coefficients ϵ_{250} = 10,980 M/cm and ϵ_{250} = 3,270 M/cm (34) were used to determine their respective concentrations. 4-carboxyaminoimidazole riboside and 5-aminoimidazole riboside were generated from stocks of CAIR and AIR (10–15 mM) treated with rAPid alkaline phosphatase (Roche) 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 × *g* for 1 min. The supernatant was transferred to a new tube and degassed for 10 min before transfer to the glove box.

Purification of AdoMet from a Pharmaceutical Source—Commercial sources of AdoMet have been found to be as little as 43% biologically active *S,S*-AdoMet (22). We found previously that, of the compounds absorbing at 259 nm, SAMe (NatureMade, Mission Hills, CA) was ~88% *S,S*-AdoMet by HPLC analysis (26). To further purify *S,S*-AdoMet, a SAMe pill was crushed, dissolved in double-distilled H_2O, and filtered through a 0.22-μm Spin-X filter (Corning). The concentration of adenine compounds was determined using the extinction coefficient ϵ_{259} = 15,400 M/cm (35), and the concentration was adjusted to 100 mM in double-distilled H_2O. 3-μl injections of the SAMe solution were separated by reverse phase-HPLC with a LC-20AT delivery system (Shimadzu, Kyoto, Japan) equipped

with a 250 × 4.6 mm Luna C18 (2), 5-μm chromatography resolution column (Phenomenex, Torrance, CA). The column was equilibrated with 90% mobile phase A (13 mM TFA) and 10% mobile phase B (methanol). The separation used a flow rate of 1 ml/min 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- to 3.85-min fraction was collected using the FRC-10A fraction collector (Shimadzu) outfitted with a extruded polystyrene foam box filled with dry ice so the purified AdoMet was immediately frozen as it was collected in a 50-ml conical tube. The purified AdoMet was lyophilized and resuspended in double-distilled H_2O sequentially three times to remove residual TFA. The purified AdoMet powder was resuspended in double-distilled H_2O (~22 mM), and samples were frozen at −20° until use. HPLC analysis determined that the purified AdoMet was 99% pure.

ThiC Activity Assays—Fpr, FldA, MTAN, AdoMet, 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 glove box. Concentrations of AIR and AdoMet 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 NADPH (0.8 mM) was added in excess, and the reaction mix was incubated for 10 min at room temperature before adding the substrate of interest. Substrates AdoMet (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 preincubated with the ThiC reaction mixture for 10 min before the relevant substrates were added. Homocysteine, aminoimidazole carboxamide, Met, adenosine, and imidazole were brought into the glove box 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 glove box. 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- to 50,000-kDa cellulose membrane with an Amicon centrifugal filter (Millipore) to remove proteins prior to transferring the samples to autosampler vials (Macherey-Nagel, Düren, 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 S.E. of the fit unless noted 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.

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

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

$$k_{cat}^0 = \frac{k[HMP]_{max}}{[\text{ThiC monomer}]} \quad \text{(Eq. 2)}$$

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

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad \text{(Eq. 3)}$$

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_{max} is the maximum velocity observed, K_{mObs} is determined by the equation $K_{mObs} = K_m(1 + [I]/K_i)$, and [S] is the concentration of substrate provided.

$$\frac{1}{v} = \frac{K_{mObs} + [S]}{V_{max}[S]} \quad \text{(Eq. 4)}$$

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_{max} is the maximum velocity, [S] is the concentration of substrate, K_s 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 α is the cooperativity factor.

$$\frac{v}{V_{max}} = \frac{\frac{[S]}{K_s}}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i} + \frac{[X]}{K_x} + \frac{[I][X]}{\alpha K_i K_x}} \quad \text{(Eq. 5)}$$

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

$$\frac{1}{v} = \frac{K_{mApp} + [S]}{V_{maxApp}[S]} \quad \text{(Eq. 6)}$$

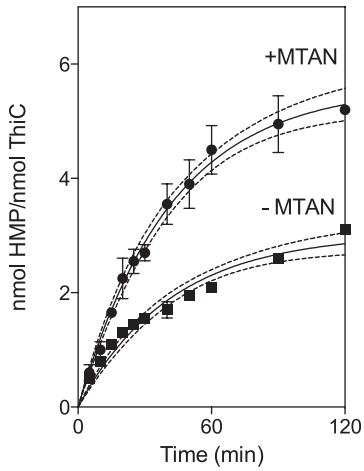

FIGURE 2. **ThiC undergoes steady-state turnover.** ThiC (0.55-nmol monomer) was incubated with flavoprotein reductase (0.5 nmol), flavodoxin A (1 nmol), NADPH (0.8 mM), AdoMet (100 μM), AIR (100 μM), and MTAN as indicated (0.1 nmol) at 37 °C. Each data point represents the mean ± S.D. of two replicates 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*.

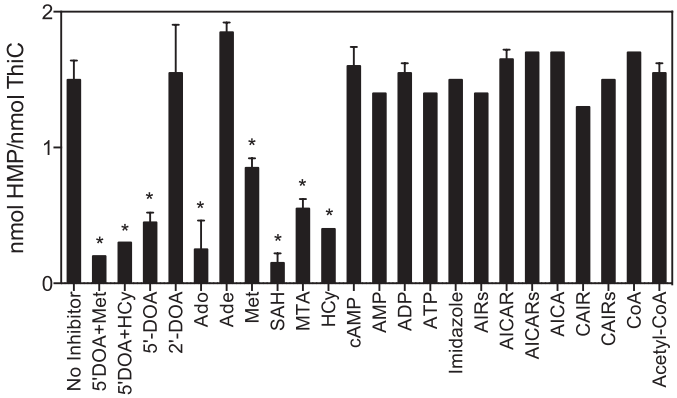

FIGURE 3. **Metabolite inhibitors of ThiC activity.** ThiC (0.55-nmol monomer) was preincubated with flavoprotein reductase (0.5 nmol), flavodoxin A (1 nmol), NADPH (0.8 mM), and potential inhibitor (0.5 mM) for 10 min at room temperature. Then AdoMet (100 μM) and AIR (100 μM) were added to initiate the reactions, which were incubated at 37 °C for 30 min. Data represent the mean ± S.D. of two replicates. The average is significantly different from the average with no inhibitor, as determined by an unpaired Student's *t* test (*, $p < 0.05$). *2′-DOA*, 2′-deoxyadenosine; *HCy*, homocysteine; *AIRs*, 5-aminoimidazole riboside; *AICARs*, aminoimidazole carboxamide riboside; *CAIRs*, 5-amino-4-imidazolecarboxylic acid riboside.

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 AdoMet (99% pure) were used in the assay. With these modifications, ThiC produced 3.1 ± 0.1 nmol HMP/nmol ThiC monomer in 2 h, confirming that multiple turnovers were possible *in vitro* (Fig. 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 mean ± S.E. of the constants determined by two independent experiments: $k_{cat}^{0} = 0.074 \pm 0.014\ min^{-1}$.

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 h. The kinetic analysis of these data yielded the turnover number $k_{cat}^{0} = 0.14 \pm 0.03\ min^{-1}$. This value for k_{cat}^{0} is within the range reported for other radical AdoMet enzymes in this class (22–24, 36, 38).

AdoMet-related Metabolites Inhibit the ThiC in Vitro Reaction—The finding that MTAN increased the reaction rate by ~2-fold suggested that 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 AdoMet-related metabolites, purines related to the substrate AIR, aminoimidazole 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).

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 AdoMet-related metabolites inhibited ThiC, specifically 5′-DOA, Met, homocysteine, adenosine (Ado), *S*-adenosylhomocysteine (SAH), and *S*-methyl-5′-thioadenosine (MTA) (Fig. 3). Of these metabolites, 5′-DOA, Met, homocysteine, and SAH are known inhibitors of radical AdoMet enzymes (reviewed in Ref. 13). The data also showed that 5′-DOA acted additively with either Met or homocysteine to further inhibit ThiC activity.

S-Adenosylhomocysteine Inhibits ThiC Competitively with AdoMet—SAH has been reported to inhibit representatives of all three classes of radical AdoMet enzymes: lysine 2,3-aminomutase, ribonucleotide reductase-activating enzyme, 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 AdoMet (25–150 μM). The Lineweaver-Burk plot of these data showed that SAH inhibited ThiC competitively with AdoMet (Fig. 4). The K_m of ThiC for AdoMet was determined by fitting data to Equation 3 from duplicate reactions of a titration of AdoMet (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 μM. On the basis of the diagnosis of competitive inhibition, the data were fit to Equation 4 using the above K_m with a global R^2 value of 0.85 and generated the kinetic constant $K_i^{SAH} = 5.6 \pm 1.1\ \mu M$.

In the cell, SAH is produced by AdoMet methyltransferases and hydrolyzed by MTAN (42). SAH is present at ~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.

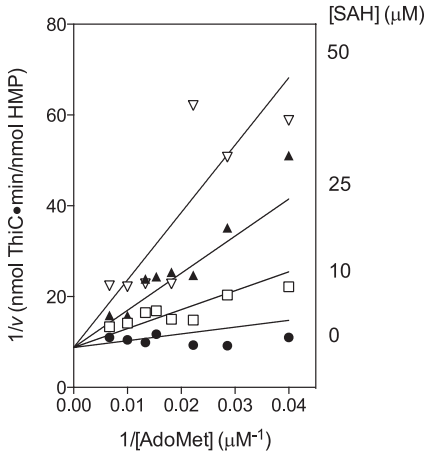

FIGURE 4. **SAH inhibits ThiC competitively with respect to AdoMet.** ThiC (0.55-nmol monomer) was preincubated with flavoprotein reductase (0.5 nmol), flavodoxin A (1 nmol), NADPH (0.8 mM), AIR (100 μM), and SAH (0, 10, 25, or 50 μM) for 10 min at room temperature. Then AdoMet (25–150 μM) was added 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.

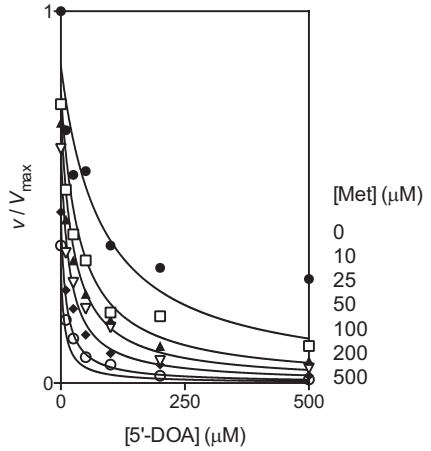

FIGURE 5. **Cooperative inhibition by 5′-DOA and Met.** ThiC (0.55-nmol monomer) was preincubated with flavoprotein reductase (0.4 nmol), flavodoxin A (1 nmol), NADPH (0.8 mM), 5′-DOA (0–500 μM), and Met (0–1000 μM) for 10 min at room temperature. Then AdoMet (100 μM) and AIR (100 μM) were added to initiate the reactions, which were incubated at 37 °C for 20 min. The data were fit to Equation 5 by non-linear regression, constraining $K_m = 17$ μM and [AdoMet] = 100 μM.

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 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 AdoMet fixed at 100 μM (Fig. 5). Dixon replots of 1/*v versus* [5′-DOA] or [Met] intersected, confirming that 5′-DOA and Met were not mutually exclusive (37). 5′-DOA and Met were assumed to inhibit competitively with respect to AdoMet. 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 $K_i^{5'-\mathrm{DOA}} = 12 \pm 2$ μM, $K_i^{\mathrm{Met}} = 82 \pm 13$ 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 h of 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 that long incubation times will not allow accurate kinetic measurements of ThiC.

Adenosine Displays Uncompetitive Inhibition with AdoMet—If adenosine bound the site occupied by the adenosine moiety of AdoMet, adenosine should also inhibit ThiC competitively with respect to AdoMet. Adenosine was added at several concentrations (0, 100, 250, and 400 μM) to reactions containing several AdoMet concentrations (25–150 μM). Unexpectedly, the data with and without adenosine resulted in parallel lines in the Lineweaver-Burk plot (Fig. 6*A*), suggesting that adenosine was uncompetitive with AdoMet and bound the ThiC-AdoMet

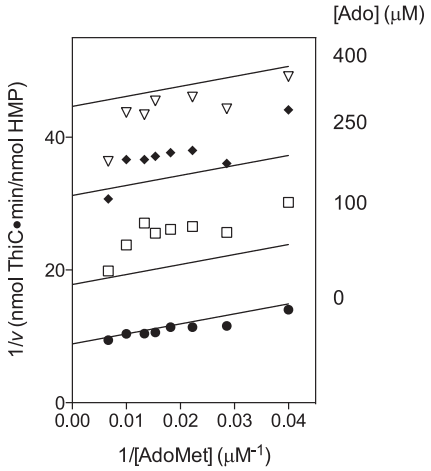

FIGURE 6. **Adenosine is uncompetitive with AdoMet inhibiting ThiC.** *A*, ThiC (0.55-nmol monomer) was preincubated with flavoprotein reductase (0.5 nmol), flavodoxin A (1 nmol), NADPH (0.8 mM), AIR (100 μM), and adenosine (0, 100, 250, or 400 μM) for 10 min at room temperature. Then AdoMet (AdoMet) (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.

complex. To determine $V_{\max}$, the [Ado] = 0 μM data were fit to Equation 3 with an 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 $K_i'^{\mathrm{Ado}} = 99 \pm 3$ μM. However, the uncertainty in the inhibition constant is likely considerably higher. We found that $K_i'^{\mathrm{Ado}}$ 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 that 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 AdoMet superfamily of enzymes. Of numerous radical AdoMet 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 here demonstrate that when product inhibition is relieved, ThiC undergoes steady-state turnover for up to 1 h.

To our knowledge, there are no other reports of radical AdoMet enzymes inhibited by adenosine or MTA, suggesting that 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 lysine 2,3-aminomutase 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 further our understanding of the radical AdoMet enzyme superfamily.

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