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Thiazole Synthase from *Escherichia coli*

*AN INVESTIGATION OF THE SUBSTRATES AND PURIFIED PROTEINS REQUIRED FOR ACTIVITY IN VITRO**[S]

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Marco Kriek[‡], Filipa Martins[‡], Roberta Leonardi[‡1], Shirley A. Fairhurst[§], David J. Lowe[§], and Peter L. Roach[‡2]

From the [‡]Chemical Biology Section, School of Chemistry, University of Southampton, Highfield, Southampton, SO17 1BJ, United Kingdom and the [§]Biological Chemistry Department, John Innes Centre, Norwich, NR4 7UH, United Kingdom

Thiamine is biosynthesized by combining two heterocyclic precursors. In *Escherichia coli* and other anaerobes, one of the heterocycles, 4-methyl-5-(β-hydroxyethyl) thiazole phosphate, is biosynthesized from 1-deoxyxylulose-5-phosphate, tyrosine, and cysteine. Genetic evidence has identified *thiH*, *thiG*, *thiS*, and *thiF* as essential for thiazole biosynthesis in *E. coli*. In this paper, we describe the measurement of the thiazole phosphate-forming reaction using purified protein components. The activity is shown to require four proteins isolated as heterodimers: ThiGH and ThiFS. Reconstitution of the [4Fe-4S] cluster in ThiH was essential for activity, as was the use of ThiS in the thiocarboxylate form. Spectroscopic studies with ThiGH strongly suggested that *S*-adenosylmethionine (AdoMet) bound to the [4Fe-4S] cluster, which became more susceptible to reduction to the +1 state. Assays of thiazole phosphate formation showed that, in addition to the proteins, Dxp, tyrosine, AdoMet, and a reductant were required. The analysis showed that no more than 1 mol eq of thiazole phosphate was formed per ThiGH. Furthermore, for each mole of thiazole-P formed, 1 eq of AdoMet and 1 eq of tyrosine were utilized, and 1 eq of 5′-deoxyadenosine was produced. These results demonstrate that ThiH is a member of the "radical-AdoMet" family and support a mechanistic hypothesis in which AdoMet is reductively cleaved to yield a highly reactive 5′-deoxyadenosyl radical. This radical is proposed to abstract the phenolic hydrogen atom from tyrosine, and the resultant substrate radical cleaves to yield dehydroglycine, which is required by ThiG for the thiazole cyclization reaction.

Thiamine (vitamin B_1) is a key nutrient for humans with a recommended daily dose of 1.4 mg, and a deficiency of thiamine causes the disease beriberi (1). Thiamine pyrophosphate (TPP)[3] is an essential cofactor for several enzymes involved in carbohydrate and amino acid metabolism, including transketolase, pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase (2, 3). Although studied for many years, the biosynthetic steps leading to TPP are not fully understood. Thiamine phosphate (TP) (Fig. 1, **6**) is formed by ThiE, an enzyme that covalently links two independently formed heterocyclic precursors, 4-amino-5-hydroxymethylpyrimidine-pyrophosphate (Fig. 1, **5**, *Hmp-PP*) and 4-methyl-5-(β-hydroxyethyl)-thiazole phosphate (Thz-P) (Fig. 1, **4**). This is converted to TPP by a ThiL-dependent phosphorylation reaction (4, 5). 4-Amino-5-hydroxymethylpyrimidine phosphate is formed by a complex rearrangement of 5-aminoimidazole ribotide by ThiC (6), which upon phosphorylation by ThiD results in 4-amino-5-hydroxymethylpyrimidine-pyrophosphate (6, 7).

The synthesis of Thz-P has been studied in a number of prokaryotes, most notably in the facultative anaerobes *Escherichia coli* and *Salmonella* sp., the aerobe *Bacillus subtilis*, and the eukaryote *Saccharomyces cerevisiae* (8, 9). Whole cell feeding studies in *E. coli* and knock-out mutants thereof indicated that Thz-P synthesis is dependent on the precursors tyrosine, cysteine, and 1-deoxy-xylulose-5-phosphate (Dxp) and the enzymes ThiFSGH, ThiI, and IscS (4, 10, 12–16). The Thz-P pathway in *B. subtilis* is significantly different, since the oxygen-sensitive iron sulfur cluster-containing enzyme ThiH is replaced by ThiO, an oxygen-tolerant FAD-dependent oxidase. ThiO utilizes glycine in the formation of dehydroglycine, the precursor that provides the C2-N3 unit of Thz-P (17). The studies of thiazole biosynthesis with purified *B. subtilis* enzymes have revealed a wealth of mechanistic insight into the action of ThiG, ThiF, ThiS, and ThiI (18–21), and a mechanism has been proposed (22), which is summarized in Fig. 2.

Far less information has been reported about the formation of the thiazole using ThiH and tyrosine. Previously, we reported that the isolation of *E. coli* ThiH was most efficient when purified from cells overexpressing ThiFSGH and that ThiH could be purified under anaerobic conditions in an apparent 1:1 complex with ThiG (14). The iron content of the as isolated ThiGH complex was less than expected for a [4Fe-4S] cluster-containing protein, although a [3Fe-4S] cluster was observed and could be reduced to yield a small amount of [4Fe-4S] cluster. The purified protein was shown to be active, since the addition of anaerobically purified ThiGH complex to a lysate of dere-

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[1] Present address: Dept. of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee 38105.

[2] To whom correspondence should be addressed: School of Chemistry, University of Southampton, Highfield, Southampton, SO17 1BJ, United Kingdom. E-mail: plr2@soton.ac.uk.

[3] The abbreviations used are: TPP, thiamine pyrophosphate; AdoMet, *S*-adenosylmethionine; Dxp, 1-deoxy-xylulose-5-phosphate; Thz-P, 4-methyl-5-(β-hydroxyethyl) thiazole phosphate; TP, thiamine phosphate; Hmp, 4-amino-5-(hydroxymethyl)-2-methylpyrimidine; FldA, flavodoxin 1; Fpr, flavoprotein:NADPH oxidoreductase; HPLC, high pressure liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; MS, mass spectrometry; DOA, 5′-deoxyadenosine.

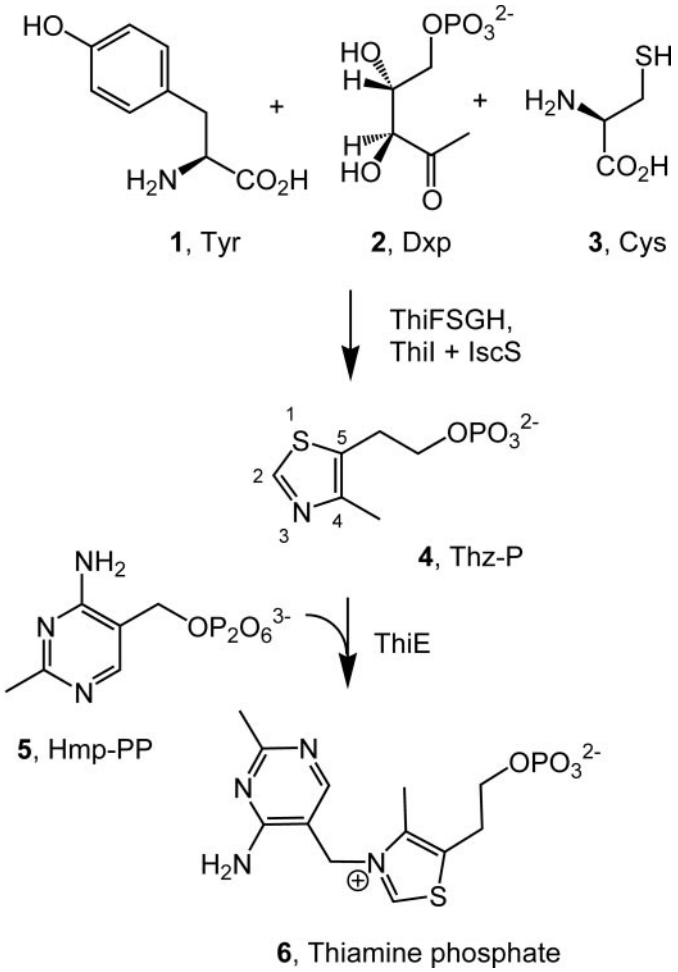

FIGURE 1. **Thiamine biosynthetic pathway in *E. coli*.** Precursors tyrosine **1**, Dxp **2**, and cysteine **3** lead to the formation of Thz-P **4**. This is coupled to 4-amino-5-hydroxymethylpyrimidine-pyrophosphate (*Hmp-PP*; **5**) to yield TP **6**.

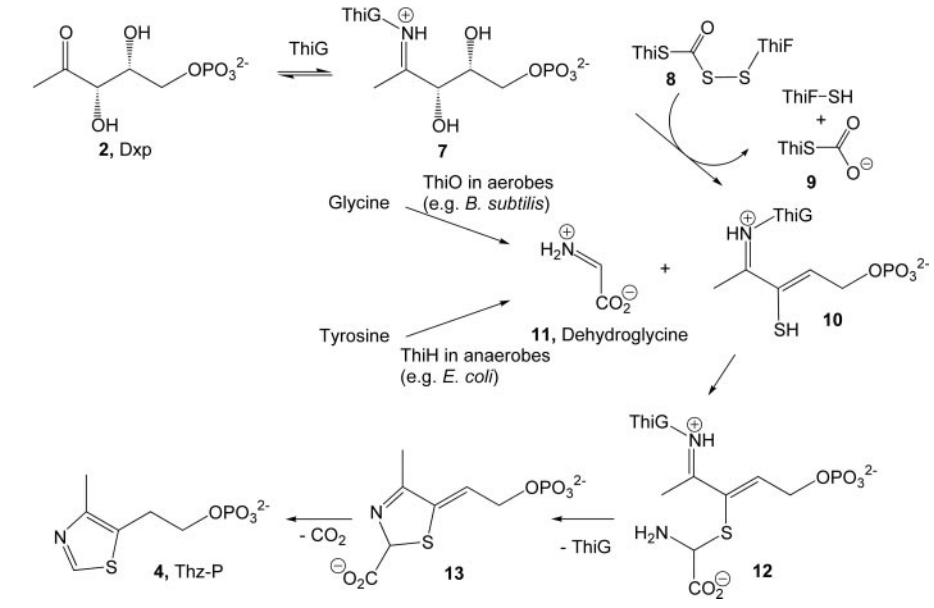

FIGURE 2. **Proposed mechanism for the formation of Thz-P.** The role of ThiG is summarized from Chatterjee *et al.* (11) and Xi *et al.* (13). Dehydroglycine **11** is proposed as the common intermediate for bacterial thiazole biosynthesis. It is derived from glycine in aerobic bacteria, such as *B. subtilis*, and from tyrosine in anaerobes, such as *E. coli*.

pressed *E. coli* 83-1, together with tyrosine, 4-amino-5-hydroxymethyl-2-methylpyrimidine (Hmp), and ATP, resulted in increased formation of thiamine (15). The relative thiamine-forming activity more than doubled upon the further addition of AdoMet, ferric chloride, sodium sulfide, and NADPH. These observations suggest the possibility of chemical iron sulfur cluster reconstitution and an AdoMet-driven reaction, most likely that of ThiH. Mutagenesis of the highly conserved cysteine-rich motif from *Salmonella enterica* ThiH suggested that these residues function as ligands to the [4Fe-4S] cluster (16).

Here we report for the first time the chemical reconstitution of the iron sulfur cluster of ThiH in the purified ThiGH complex. Spectroscopic studies demonstrate the binding of AdoMet to the reconstituted cluster. We show that Thz-P can be synthesized *in vitro* using reconstituted ThiGH, an anaerobically purified complex of ThiFS, and a combination of flavodoxin, flavoprotein reductase, and NADPH as a reducing system. In addition, three substrates were required: tyrosine, AdoMet, and Dxp. The Thz-P formed in this reaction was measured in a coupled assay, by adding ThiDE and Hmp to form TP, which was oxidized to the fluorescent thiochrome derivative (23). These measurements showed that ThiGH was not catalytic in the *in vitro* assay, but up to 1 mol eq of Thz-P was formed per mole of ThiGH present in the assay. The dependence of the Thz-P-forming reaction on these substrates and the concomitant formation of DOA confirmed that ThiH belongs to the "radical-AdoMet" superfamily.

EXPERIMENTAL PROCEDURES

Materials—Reagents and materials were obtained from the following suppliers: far UV HPLC grade acetonitrile and anhydrous $FeCl_3$ from Acros (Loughborough, UK); MOPS, AdoMet hydrochloride salt, FMN, FAD, phenylmethylsulfonyl fluoride, sodium sulfide nonahydrate, lysozyme, Kodak Biomax MR single emulsion autoradiography film, and oligonucleotides from Sigma; benzonase and pET-24d(+) from Novagen (Merck); 20% perchloric acid from Fluka (Poole, UK); L-[U-^{14}C]tyrosine, *S*-adenosyl-L-[*methyl*-^{14}C]methionine, and NAP-10 gel filtration columns from GE Healthcare; acetic acid, potassium acetate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and TLC plates (either aluminum-backed silica Gel-60 (F-254, 250 μm) or RP-18 F254) from BDH-Merck; arabinose from Alfa-Aesar (Heysham, UK); DTT, isopropyl 1-thio-β-D-galactopyranoside, chloramphenicol, ampicillin, and kanamycin from Melford Laboratories (Ipswich, UK); film developer CEATANK and fixative CEAFIX from A. Somerville (Huddersfield, UK). Molecular biology reagents were obtained from the following

TABLE 1
Plasmids constructed or used in this study

Plasmid	Insert	Parent plasmid	Restriction sites	Reference
pMK060	N-terminal His_6 tag	pBADHisA		This study
pRL1020	*E. coli thiFSGH*	pBADHisA	NcoI/XhoI	Ref. 14
pMK131	*B. subtilus thiE*	pET24d(+)	NcoI/XhoI	This study
pMK141	*E. coli thiD*	pET16b	NdeI/BamHI	This study
pMK171	*E. coli thiFS*	pMK060	NcoI/XhoI	This study
pFLAV	*E. coli fldA*	pET24d(+)	NcoI/BamHI	Ref. 25
pFLDR	*E. coli fpr*	pET24d(+)	NcoI/BamHI	Ref. 25
pT-groELS	*E. coli groELS*			Ref. 24

suppliers: Pfu polymerase from Stratagene (Amsterdam, The Netherlands), pBadHis from Invitrogen, and restriction enzymes from New England Biolabs (Hitchin, UK). Tryptone and yeast extract were purchased from Oxoid (Fisher). *B. subtilis* DNA was a kind gift from Prof. P. Glaser (Institute Pasteur, Paris, France). pT-GroESL was a kind gift from Dr. M. Sami (Oxford University) (24), and pFLAV and pFLDR are pET24d(+) derivatives that express flavodoxin 1 (FldA) and flavoprotein:NADPH oxidoreductase (Fpr), respectively (25), and were a kind gift from Dr. K. S. Hewitson (Oxford University).

Methods—All protein purifications, protein reconstitutions, and assays were maintained under nitrogen in an anaerobic glove box with less than <2 ppm of O_2 (Belle Technology, Portesham, UK) equipped with an Amersham Biosciences Acta fast protein liquid chromatograph and VC130 sonicator (Sonics and Materials, Newtown, CT). Cell lysates were cleared in 250-ml gas-tight polycarbonate centrifuge bottles (Beckman-Coulter, High Wycombe, UK). A Gilson 321 HPLC work center equipped with a dual wavelength UV-visible detector and a Shimadzu RF-10Axl fluorescence detector was used for analytical HPLC methods; chromatograms were collected and analyzed using the Gilson Unipoint software (Gilson). Mass spectrometry analysis was carried out using a Gilson HPLC coupled to a Thermo Finnigan Surveyor MSQ single quadrupole mass spectrometer with electrospray ionization. The data were collected and processed using the XCalibur software system, and multiple charged species were analyzed by Promass Deconvolution (Thermo, Altrincham, UK).

Spectroscopy—EPR spectra were recorded at X-band on a Bruker ELEXSYS 500 spectrometer with an ER049X microwave bridge using an ER4122SHQ cavity. The experiments at low temperature used an ESR 900 liquid helium flow cryostat and ITC3 temperature controller (Oxford Instruments). Cluster concentration was estimated by double integration of spectra by comparison with a 1 mM Cu(EDTA) standard. UV-visible spectra of ThiGH (1 mg/ml) were recorded with an Ocean Optics (Duiven, The Netherlands) USB2000 spectrophotometer using a light source Mini-D2-GS connected by optical fibers P-400-2-UV/SR to a cuvette holder inside the glove box.

Chemical Synthesis—Hmp and 4-methyl-5-(β-hydroxyethyl) thiazole phosphate (Thz-P) were prepared as described previously (26, 27). Dxp was synthesized as described by Taylor *et al.* (28) and purified as described by Leonardi and Roach (15).

Plasmid Construction—A plasmid pMK060 was constructed to allow N-terminal His_6 tag extended proteins to be expressed under the control of a pBAD promoter. Using PCR, a sequence encoding a new N-terminal His tag was added by the insertion of CC**ATG**CACCACCACCACCACCATG after the A at position 317 of pBAD-His, immediately followed by the original NcoI site. The new start codon is shown in boldface type and yields proteins with an N-terminal Met-His_6-Ala sequence. *B. subtilis* ThiE was amplified to include a N-terminal Met-Ala-His_6 tag and inserted into pET24d(+), yielding pMK131. The open reading frames were amplified by PCR, the purified PCR products were restricted, and the resultant fragments were ligated into a suitably restricted parent plasmid to yield the product plasmids shown in Table 1. The PCR amplification of ThiFS introduced an NcoI restriction site and modified the N terminus of ThiF to introduce an addition of Val after the natural Met start codon.

Protein Expression—Proteins were expressed in *E. coli* BL21(DE3) cells transformed with the required plasmid. Cells were grown at 37 °C in Fernbacher flasks (4 liters) containing 2YT medium (1.25 liters) with the required antibiotic (ampicillin (100 mg/liter), kanamycin (34 mg/liter), chloramphenicol (25 mg/liter)), using isopropyl 1-thio-β-D-galactopyranoside (1 mM) or arabinose (2 g/liter) induction when the $A_{600\ nm}$ reached 0.60. The induced cultures were grown for a further 4 h at 27 °C, and cells were harvested by centrifugation. FldA was expressed from BL21(DE3) cells transformed with pFLAV as described previously (25) using growth medium fortified with FMN (2 mg/liter). Fpr was co-expressed with GroESL in *E. coli* BL21(DE3) cells transformed with pFLDR and pT-groESL (24, 25).

Anaerobic Protein Purification—All protein concentrations were determined by the method of Bradford (29) using bovine serum albumin for calibration, and protein purity was assessed by SDS-PAGE using 15% acrylamide gels (30). Purified proteins were stored at −80 °C until use. A general protocol for the anaerobic purification of His_6-tagged proteins was used and is described below with protein-specific modifications described afterward. Inside the anaerobic glove box, cell paste (30 g) was resuspended in anaerobic buffer A (100 ml, 50 mM MOPS, 200 mM NaCl, 50 mM imidazole, 10% (v/v) glycerol, pH 7.7), to which was added lysozyme (10 mg) and benzonase (20 μl). The cell suspension was stirred for 1 h at room temperature followed by sonication (30× 1-s burst (20 W)/min for 30 min) in a flask cooled in a water bath (~10 °C) inside the glove box. The lysate was cleared by centrifugation (Beckman JA-14; 13,000 rpm, 30 min, 4 °C), and the cleared lysate was applied to a nickel-charged chelating Sepharose column (30 ml) previously equilibrated in anaerobic buffer A (200 ml). The column was washed with 150 ml of buffer A, and then the protein was eluted using an imidazole gradient to 250 mM over 50 ml using buffer C (buffer A plus 500 mM imidazole) followed by a 100-ml iso-

cratic wash. The desired protein fractions (30-ml maximum) were pooled and desalted on a 50-ml Superdex S-75 column pre-equilibrated in buffer D (50 mM MOPS, 100 mM NaCl, 10% (v/v) glycerol). The protein fractions (10 ml) were stored at −80 °C in sealed 15-ml Falcon tubes. For ThiGH, the general protocol was modified by the addition of phenylmethylsulfonyl fluoride (16 mg in 1 ml of isopropyl alcohol) to the lysis buffer. After applying the protein to the nickel-charged Sepharose column, the column was washed with 100 ml of buffer B (buffer A plus 500 mM NaCl) followed by 50 ml of buffer A. For the Superdex 75 column, 5 mM DTT was added to buffer D. In the purification of *E. coli* ThiD, a second gradient (50 ml) was used for the nickel-charged column to reach a higher imidazole concentration (500 mM), followed by an isocratic wash (100 ml). The protocol was modified for ThiFS by eluting from the nickel-charged column with a gradient to a lower imidazole concentration (125 mM).

Aerobic Protein Purifications—Fpr and FldA were purified under aerobic conditions at 4–7 °C and stored at −80 °C. Fpr was purified as follows. Cells with expressed Fpr were stirred aerobically in buffer E (50 mM Tris, 10% (v/v) glycerol, pH 8.1, lysozyme (200 mg/liter), and benzonase (200 μl/liter)) at room temperature for 30 min. The suspension was sonicated on ice using 30 bursts of 30 s in 30 min. The lysate was cleared by centrifugation (Beckman JA14; 13,000 rpm) and applied to a 200-ml Q-Sepharose column pre-equilibrated in buffer F (50 mM Tris/HCl, pH 8.1), washed with buffer F, and eluted with a 500-ml gradient to 40% buffer G (buffer F plus 1 M NaCl). The yellow fractions were pooled, and ammonium sulfate was added to a final concentration of 2 M. A Phenyl Source column (200 ml) was pre-equilibrated in buffer H (buffer F plus 2 M ammonium sulfate), and protein was eluted with a gradient over 500 ml to 100% buffer F. The yellow fractions were pooled, concentrated, and further purified on a Superdex S-75 column (33 × 850 mm) equilibrated in buffer F. Fpr-containing fractions were pooled and concentrated to 1.4 mM. FldA was purified by a method similar to that described by Osborne *et al.* (31). Purified FldA (1 ml, 1.38 mM) was reconstituted by incubation with an equimolar concentration of FMN (10 μl, 140 mM) for 2 h at 37 °C. Excess FMN was removed by applying the solution to an NAP-10 gel filtration column pre-equilibrated in buffer F (20 ml).

Reconstitution and Concentration of ThiGH—DTT solution in buffer D (25 μl, 200 mM) was added to a sample of purified ThiGH complex (1 ml, 83 μM) and gently mixed. After 15 min, 5 mol eq of $FeCl_3$ in water (40 μl, 10 mM) were added carefully in small aliquots (5 μl), and after a further 10 min, 5 mol eq of Na_2S in water were added likewise (total added 40 μl, 10 mM). The protein solution was incubated for another 2 h, and then the precipitated iron sulfide was removed by applying the solution to a NAP-10 gel filtration column pre-equilibrated and eluted with buffer D. The iron content was analyzed by the methods of Fish (32). Reconstituted ThiGH could be concentrated to a maximum concentration of 40 mg/ml using a Biomax 5000 molecular weight cut-off Ultrafree 0.5-ml centrifugal filter (Millipore, Watford, UK).

Radiochemical Assay with L-[U-^{14}C]Tyrosine—For experiments with radiolabeled substrates, ThiGH was purified as described previously (14) and reconstituted. To samples of as isolated or reconstituted ThiGH (final concentration 55 μM) was added AdoMet (1.68 mM), FldA (12 μM), Fpr (61 μM), and NADPH (1.6 mM), followed by [U-^{14}C]tyrosine (8.9 μM, 0.5 μCi) in a total volume of 124.5 μl. This was divided into 20-μl aliquots and incubated at 37 °C for 5, 15, 30, 60, 120, or 480 min. Negative control experiments were prepared that lacked AdoMet but were otherwise treated identically to the normal assays. At the indicated time points, the samples were frozen (−80 °C). Reactions were stopped by thawing in air and were analyzed by spotting samples (1 μl) onto normal phase TLC plates. These plates were developed in *n*-propanol/ethyl acetate/water (6:1:3) and dried in air, and radioactivity was monitored by autoradiography using films exposed for 16 h. The following R_f values were obtained in this solvent system for unlabeled standards: 4-hydroxybenzyl alcohol (0.92), tyrosine (0.61), and glyoxylic acid (0.31).

Radiochemical Assay with [methyl-^{14}C]AdoMet—To solutions of as isolated or reconstituted ThiGH (final concentration 62 μM) was added FldA (21 μM), Fpr (4 μM), NADPH (0.93 mM), tyrosine (925 μM), and [*methyl*-^{14}C]AdoMet (47 μM, 0.25 μCi) in a total volume of 107 μl. This was divided into 10-μl aliquots. Negative control experiments were prepared that lacked tyrosine but were otherwise treated identically to the normal assays. The aliquots were incubated at 37 °C for 0, 30, 60, 120, 240, and 480 min prior to freezing (−80 °C). Samples were thawed in air and directly spotted onto a reverse phase TLC plate (2 μl). The TLC plates were developed in acetonitrile/buffer H in a ratio of 1:3, where buffer H is an aqueous solution of acetic acid (25 mM) and octanesulfonic acid (8 mM). TLC plates were air-dried, and the radioactivity was monitored by autoradiography using films exposed for 16 h.

EPR Studies of Reconstituted ThiGH—Two aliquots of anaerobically purified ThiGH (1 ml, 133 μM) were each incubated with DTT (25 μl, 200 mM) for 10 min, $FeCl_3$ (33 μl, 20 mM) for 10 min, and Na_2S (39.6 μl, 20 mM) for 90 min. The mixtures were centrifuged (Stratagene Picofuge, maximum speed) to remove precipitated protein and excess iron sulfide. The concentration of reconstituted ThiGH in the resultant solution was estimated to be 69 μM. Reagents were added to a sample of reconstituted ThiGH (250-μl fractions in duplicate) in the order shown to give the following final concentrations: buffer (negative control), AdoMet (1.67 mM), tyrosine (1.77 mM), AdoMet (1.67 mM) plus tyrosine (1.77 mM), or sinefungin (1.77 mM). One of each duplicate sample was reduced by the addition of dithionite (0.9 mM). Samples of these solutions (200 μl) were frozen and stored in liquid nitrogen in quartz EPR tubes. Spectra were recorded under the following conditions: temperature, 10 K; microwave frequency, 9.4340 GHz; microwave power, 2 milliwatts; and field modulation amplitude, 0.4 milliteslas.

HPLC Assay for TP as Thiochrome—TP-containing solutions were oxidized with an alkaline solution of $K_3(Fe(CN)_6)$, neutralized, separated by HPLC, and analyzed by fluorescence detection as described previously (15).

HPLC-MS Analysis of ThiGH Assays and Protein Samples—The eluate from the HPLC column was split in a 1:4 ratio, and the main flow was sent to a dual wavelength absorbance detector set at 215 and 260 nm. The minor flow was diluted with

make-up solvent from a third pump, which added acetonitrile/H_2O with 0.3% formic acid at 0.5 ml/min. This flowed into the mass spectrometer and was analyzed in ES+ mode. DOA was detected using a Synergy Polar-RP column (4 μm, 80 Å, 150 × 4.6 mm) with the following solvents: solvent A was water with 0.1% formic acid, adjusted to pH 3.8 with NH_4OH; solvent B was 90% (v/v) acetonitrile, 10% (v/v) H_2O. The column was equilibrated in solvent A at a flow rate of 0.8 ml/min, and the sample was injected, after which the following elution profile was applied: 6 min isocratic (100% solvent A) and then a 20-min gradient to 50% solvent A, followed by a 5-min gradient to 10% solvent A, 5 min isocratic at 10% solvent A, and a 4-min gradient to 100% solvent A, 15 min isocratic at 100% A. DOA and tyrosine were measured in a sample using a Phenomenex ODS (3) Prodigy (5 μm, 100 Å, 250 × 4.6 mm) column equilibrated and solvents C (0.1% aqueous trifluoroacetic acid) and D (acetonitrile, 0.1% trifluoroacetic acid) using the same elution profile. Under these conditions, tyrosine and DOA eluted with a retention time of 17 and 17.4 min, respectively. The ES+ ions were quantified using a calibration of standards of known concentration. Before analysis, protein samples were first desalted by NAP-10 buffer exchange into ammonium bicarbonate buffer (10 mM), and then a sample was injected onto a Vydac Protein C4 analytical column using 0.1% trifluoroacetic acid in water for solvent A and 9:1 acetonitrile/water with 0.1% trifluoroacetic acid for solvent B. The proteins were eluted with a gradient from 10 to 90% solvent B over 50 min at a flow of 1.0 ml/min.

Standard ThiGH Activity Assay—A full protocol is described here, but below, only the modifications are described. The following proteins and reagents were added to a reaction mixture in the following order (with final concentrations): NADPH (1 mM), FldA (92 μM), Fpr (16.5 μM), reconstituted and concentrated ThiGH (87 μM), ThiFS (106 μM), Na_2S (266 μM), Dxp (2 mM), tyrosine (2 mM), AdoMet (1.8 mM), ThiD (4.6 μM), ThiE (11.6 μM), Hmp (653 μM), ATP (3.3 mM), and $MgCl_2$ (6.6 mM). The reaction was divided into 200-μl aliquots, which were incubated at 37 °C for 5, 15, 30, 45, 60, and 120 min and subsequently stored frozen (−80 °C). A standard method was developed for the analysis of these supernatants as follows. Samples were thawed and proteins were immediately precipitated with 20% perchloric acid (12 μl) and then cleared by centrifugation (Eppendorf 5415D microcentrifuge, maximum speed), and the supernatants were analyzed by either HPLC-MS for AdoMet, tyrosine, and DOA (100 μl) or using the thiochrome assay (20 μl) for thiamine.

Effect of Varying Protein Concentrations on ThzP Formation—The standard assay was modified as follows: NADPH (0.75 mM), FldA (69 μM), Fpr (12.3 μM), ThiGH (65 μM), ThiFS (96 μM), Na_2S (200 μM), Dxp (1.5 mM), tyrosine (1.5 mM), and AdoMet (1.35 mM). When conversion of Thz-P to TP was required, ThiD (3.4 μM), ThiE (8.7 μM), Hmp (490 μM), ATP (2.5 mM), and $MgCl_2$ (4.9 mM) were also added. Protein concentrations were varied by preparing samples containing additional ThiGH (final concentration 130 μM) and another containing additional ThiFS (final concentration 191 μM).

Effect of ThiGH Substrates on TP Formation—The standard assay was modified as follows: reconstituted and concentrated ThiGH (116 μM), ThiFS (110 μM). Substrate concentrations were varied as follows: tyrosine (0–0.5 mM), AdoMet (0–0.5 mM), and Dxp (0–1.0 mM). The Thz-P formed in the reaction was converted to TPP and quantified as the thiochrome (23).

HPLC-MS Analysis of a ThiGH Assay—The standard assay was modified as follows: ThiGH (255 μM), tyrosine (0.51 mM), AdoMet (0.74 mM), NADPH (0.67 mM), FldA (62 μM), and Fpr (10 μM). The samples were analyzed by HPLC-MS.

TABLE 2
Mass spectrometric analysis of thiazole biosynthetic enzymes

Protein sample	Observed masses	Calculated masses	Comment
	Da	*Da*	
ThiGH	44,145.0 ± 3.1	44,143.0	ThiH-His_6
	26,894.8 ± 1.8	26,896.1	ThiG
	25,515.5 ± 1.9	25,512.6	ThiG residues 1–242
ThiFS	7327.7 ± 0.5	7327.0	ThiS thiocarboxylate
	28,090.9 ± 2.3	28,094.0	ThiF
ThiD	31,042.0 ± 0.7	31,023.1	ThiD
ThiE	24,568.0 ± 0.5	24,574.9	*B. subtilis* ThiE

RESULTS AND DISCUSSION

Protein Isolation and Reconstitution—The protein fractions used in the ThiGH activity assays were of high purity as indicated by the SDS-polyacrylamide gel visualized with Coomassie stain (see supplemental Fig. S1). Anaerobic purifications of the ThiGH complex from pRL1020/BL21(DE3) in MOPS buffer, pH 7.7, resulted on average in 2.7 mg of purified protein complex/g of wet cell paste (average from 15 purifications). Mass spectrometry was used to further characterize the protein samples. In addition to the expected masses for His-tagged ThiH and ThiG, a mass corresponding to the loss of the C-terminal 14 residues of ThiG was also observed (Table 2). By SDS-PAGE, a weak band that may correspond to this species can be seen just below the ThiG band (Fig. S1, *lane 2*). Attempts to use protease inhibitors during the purification to reduce this apparent modification were unsuccessful.

The anaerobic purification of ThiFS complex from pMK171/BL21(DE3) resulted in a protein yield of 3.6–4.9 mg/g of wet cell paste. The protein was characterized by mass spectrometry (Table 2), and the observed masses suggested that ThiS had be isolated as the previously observed C-terminal thiocarboxylate form (12, 13). SDS-PAGE analysis of ThiFS (Fig. S1) showed that ThiS is poorly visualized by Coomassie stain, but when a more concentrated sample (2.6 mg/ml) was applied to gel, a band with the expected protein size is visible (Fig. S1, *lane 9*). The yield of purified *E. coli* ThiD varied from 0.5 to 1.7 mg/g of wet cell paste. The large scale production of soluble *E. coli* ThiE (5) proved difficult, but the use of *B. subtilis thiE*, which is functionally equivalent to the *E. coli* gene, resulted in soluble, active protein when expressed in *E. coli* and yielded 22 mg of protein/g of wet cell paste.

The amount of iron present in the ThiGH complex purified from pRL1020/Bl21(DE3) under anaerobic conditions was typically 1.0 ± 0.4 mol eq of iron/mol of ThiH but was occasionally higher (up to 2 eq of iron). After reconstitution and gel filtration, the iron content was measured as 5.2 ± 1.3 mol eq of iron/mol of ThiH, suggesting that some residual adventitiously bound iron may remain in these samples. Optimization of the

reconstitution of the iron sulfur cluster in ThiGH identified several important factors. A relatively short incubation period with fresh DTT solution for 15 min was better than for 60 min, since the latter condition resulted in protein precipitation upon the subsequent addition of $FeCl_3$. The $FeCl_3$ solution was freshly prepared from black anhydrous $FeCl_3$, whereas the use of brown caked (wet) $FeCl_3$ did not result in successful reconstitution and resulted in protein precipitation upon concentration. Upon the addition of $FeCl_3$, the protein solution turned red, which became brown over 15 min, at which point Na_2S was added in small aliquots (one-half eq at a time) followed by gentle mixing. After 2 h, the resultant solution was gel-filtered through a NAP-10 column to remove excess iron and precipitated iron sulfide and then cautiously concentrated to avoid precipitation, routinely resulting in solutions of 20–40 mg/ml (0.3–0.6 mM), which were used directly for further experiments.

Spectroscopic Studies of ThiGH—UV-visible absorbance spectra of reconstituted ThiGH (Fig. 3) are in broad agreement with those reported earlier for as isolated ThiGH (14) but showed a stronger absorbance between 300 and 450 nm. The extinction coefficient for reconstituted ThiGH at 390 nm was 4678 $cm^{-1}\ M^{-1}$/iron atom (assuming a 4Fe-4S cluster).

The EPR spectrum of reconstituted ThiGH reduced with dithionite had a $g_{\|} = 2.03$ and $g_{\perp} = 1.92$ integrating to 16.7 μM from 63.7 μM protein complex present (Fig. 4). This spin concentration is a marked increase relative to the reduced spectrum of as isolated protein reported previously, which integrated to ~2% of the ThiGH concentration (14). The difference in signal strength may explain the slight shift in the g values from those obtained previously ($g_{\|} = 2.02$ and $g_{\perp} = 1.92$), but both sets of g values are within the range expected for a $[4Fe\text{-}4S]^{1+}$ cluster. Ollagnier *et al.* (33) reported a $g_{\|} = 2.026$ and $g_{\perp} = 1.93$ signal assigned to $[4Fe4S]^{1+}$ in dithionite-reduced ribonucleotide reductase. The addition of tyrosine did not produce a marked change in the EPR spectrum, whereas the addition of AdoMet had a major effect (Fig. 4*C*). The simplest explanation for this observation would be that the AdoMet had bound to the iron sulfur cluster and enhanced the susceptibility of the cluster to reduction (Fig. 5, **15** → **16**). Changes of reduction potentials for $[4Fe\text{-}4S]^{2+/1+}$ have been observed for other members of the radical-AdoMet family, such as anaerobic ribonucleotide reductase-activating enzyme (34) and lysine aminomutase (35, 36). The EPR signal measured upon the addition of AdoMet corresponded to a concentration of 57.8 μM, which is consistent with 90% of the protein being reduced. The main features of the spectrum were typical of a $[4Fe\text{-}4S]^{1+}$ cluster with $g_{\|} = 2.00$ and $g_{\perp} = 1.87$. Broderick *et al.* (37) reported a similar spectrum for dithionate-reduced pyruvate formate-lyase-activating enzyme in the presence of AdoMet.

In comparison, a ThiGH sample that had both AdoMet and tyrosine added showed a much weaker EPR signal. These observations are consistent with the added tyrosine reacting with the AdoMet bound to the reduced cluster, possibly through the mechanism shown in Fig. 5 (**1** → **18**). The reaction of tyrosine with a sample of reduced AdoMet·ThiGH complex was very fast, and 1 min after adding the tyrosine, the strong $[4Fe\text{-}4S]^{1+}$ signal had almost disappeared. The rapid disappearance of the

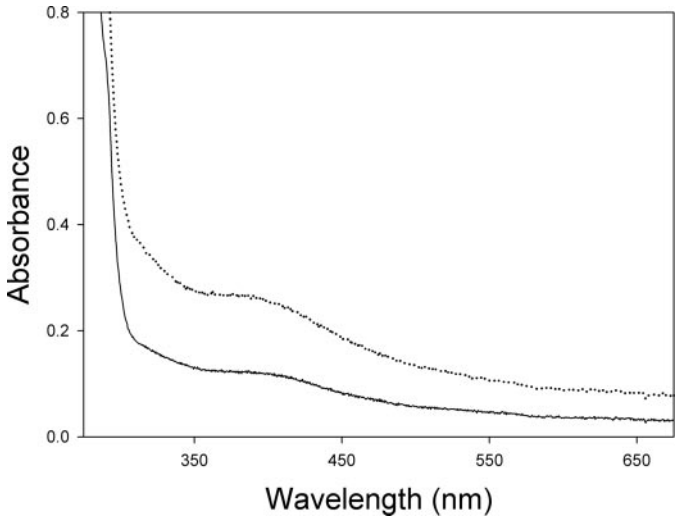

FIGURE 3. **Absorbance spectra of ThiGH samples.** Anaerobically purified ThiGH (1 mg/ml protein, 14 μM ThiH) was incubated with 5 mM DTT (15 min), 5 mol eq of $FeCl_3$ (15 min), and 5 mol eq of Na_2S (2 h). *Heavy line*, before reconstitution; *light line*, after reconstitution.

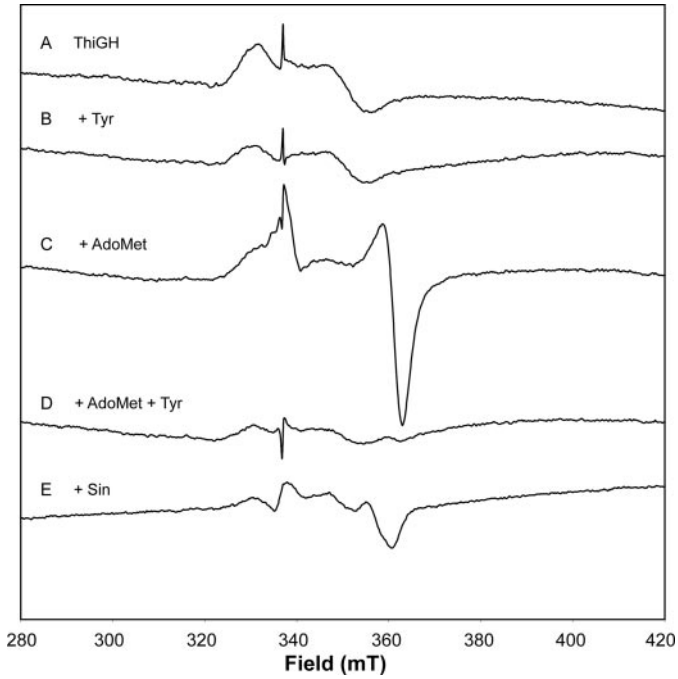

FIGURE 4. **X-band EPR spectra of ThiGH.** All samples are background-subtracted using an unreduced sample. Samples contained reconstituted ThiGH reduced with 1 mM dithionite for 30 min and frozen in liquid N_2. *A*, reconstituted ThiGH; *B*, *A* plus tyrosine; *C*, *A* plus AdoMet; *D*, *A* plus tyrosine and AdoMet; *E*, *A* plus sinefungin. The sharp spike at ~338 milliteslas (*mT*) in each spectrum is a background signal.

$[4Fe\text{-}4S]^{1+}$ cluster signal upon the addition of tyrosine in the EPR experiments shows that, at least under these conditions, the reaction with tyrosine is faster than the rate at which the cluster can be converted back to the reduced state.

Samples of ThiGH reduced in the presence of sinefungin, a structural analog of AdoMet (38), showed (Fig. 4*E*) similar changes to the spectrum of AdoMet (Fig. 4*C*). In the sinefungin case, a smaller proportion of the $[4Fe\text{-}4S]^{1+}$ cluster with $g_{\perp} = 1.87$ was observed. This indicates that AdoMet and sinefungin are both able to bind to the same site. Finally, attempts to

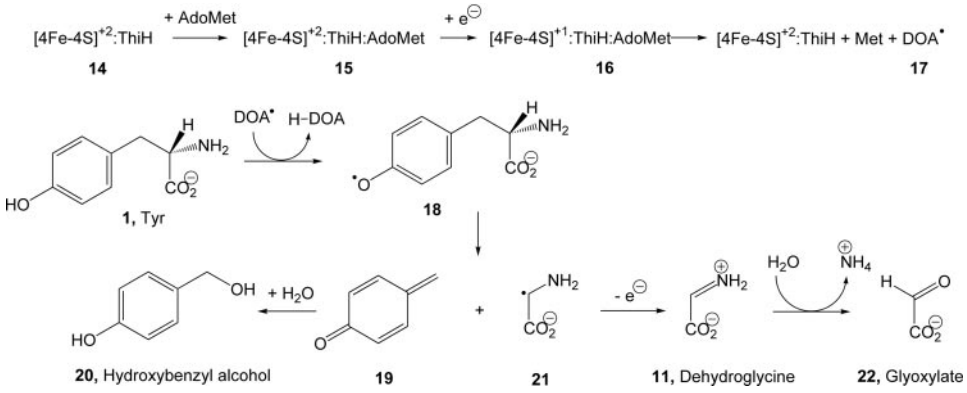

FIGURE 5. **Proposed mechanism for ThiH-dependent cleavage of tyrosine.** ThiH mediates the reductive cleavage of AdoMet to yield a deoxyadenosyl radical, which can abstract the phenolic hydrogen atom from tyrosine. The resultant radical undergoes C_{α}–C_{β} bond cleavage to yield the quinone methide **19** and a glycinyl radical **21**. A one-electron oxidation of **21** yields dehydroglycine **11**, which is a substrate for ThiG-dependent Thz-P formation. Alternatively, during *in vitro* assays that lack Dxp or the sulfur donor, **11** can undergo hydrolysis to glyoxylate **22**.

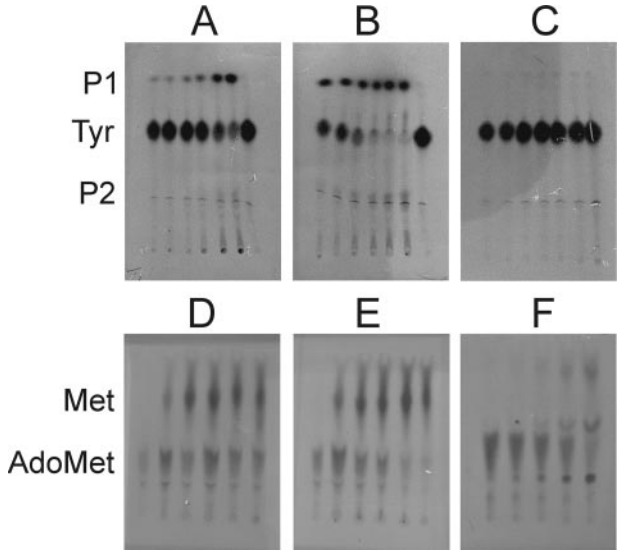

FIGURE 6. **Autoradiography of ThiGH Assays.** Assays contained either L-[U-^{14}C]-tyrosine (*A–C*) or [^{14}C-Methyl]-AdoMet (*D–F*). Six time points are spotted onto each plate (5, 15, 30, 60, 120, or 480 min (*left* to *right*)), followed by a standard. *A*, as isolated ThiGH, [U-^{14}C]tyrosine, and AdoMet; *B*, reconstituted ThiGH, [U-^{14}C]tyrosine, and AdoMet; *C*, reconstituted ThiGH and [U-^{14}C]tyrosine; *D*, as isolated ThiGH, tyrosine, and [methyl-^{14}C]AdoMet; *E*, reconstituted ThiGH, tyrosine, and [*methyl*-^{14}C]AdoMet; *F*, reconstituted ThiGH and [*methyl*-^{14}C]AdoMet.

observe the putative tyrosine-derived radical intermediate (Fig. 5, **18**) were unsuccessful, but this may simply indicate that the kinetics do not favor the accumulation of this intermediate.

Reaction of Radiochemically Labeled ThiH Substrates—The activity of ThiGH was investigated using two radiochemically labeled substrates, L-[U-^{14}C]tyrosine and *S*-adenosyl-L-[*methyl*-^{14}C]methionine. The assays were analyzed by TLC and autoradiography (Fig. 6). The results show some activity for as isolated ThiGH (Fig. 6, *A* and *D*), but the reconstituted protein has much greater activity with both substrates (Fig. 6, *B* and *E*). Artifacts arising from the instability of the substrates (particularly AdoMet (39)) were excluded through control experiments that were identical to the full assay except that they lacked the reducing system (Fig. 6, *C* and *F*). The turnover of tyrosine led to the formation of two products, P1 and P2 (Fig. 5), which were tentatively assigned as 4-hydroxybenzyl alcohol and glyoxylic acid (Fig. 5, **20** and **22**, respectively) on the basis of the R_f values of authentic standards. These compounds could arise by reaction of the initially formed products, quinone methide **19** and dehydroglycine **11**, with water. Therefore, we propose that dehydroglycine **11** is the intermediate at which the aerobic and anaerobic microbial pathways converge. The experiments also showed that the reaction is mutually dependent on AdoMet and tyrosine; the absence of either AdoMet or tyrosine stopped the turnover of the other substrate.

Time Course of Thz-P Formation—An assay mixture capable of measuring ThiGH activity was developed by combining the required components. The highly polar Thz-P is difficult to quantify directly by reverse phase HPLC, but Thz-P is readily converted to TP in an enzyme-coupled reaction with HMP, ATP, ThiD, and ThiE. The TP can then be quantified as the fluorescent thiochrome derivative (23). The conditions for the efficient enzymatic conversion of Thz-P to TP were optimized using a synthetic sample of Thz-P to ensure this step was not rate-limiting (data not shown). The coupled assay was then assembled, containing reconstituted ThiGH, ThiFS, tyrosine, AdoMet, Dxp, FldA, Fpr, and NADPH as the reducing system and gave an initial rate for TP formation of 40 μM/h (Fig. 7) with a final amount of TP formed equal the amount of ThiGH present, 87 μM. This represents a single turnover of ThiGH to form Thz-P, and extended incubation did not result in the formation of more reaction product (data not shown). In subsequent experiments, some variation in the amount of Thz-P formed was observed, but this was in the range 0.5–1 mol eq relative to ThiGH.

During the initial optimization of the assay for Thz-P-forming activity, the effect of dithionite as a reducing system was investigated, but it resulted in no measurable activity. This result was initially somewhat surprising, since dithionite had proved a satisfactory reductant for the EPR experiments with ThiGH. However, the Thz-P-forming reaction is also dependent upon ThiFS, which may also make use of a redox-sensitive intermediate. In particular, Begley and co-workers (13) have observed an interchain acyl persulfide of ThiFS and have shown that the S–S bond is formed between the C-terminal thiocarboxylate of ThiS and residue Cys^{184} of ThiF. Their studies of the ThiF Cys^{184} → Ser mutant have demonstrated that although the mutant could efficiently form the ThiS thiocarboxylate, it was unable to complement an *E. coli* (thiF^{-}) strain. These results therefore imply the involvement of the acyl persulfide in thiazole biosynthesis. It may be that the addition of dithionite to our ThiGH assays lead to the reduction of the acyl persulfide, and once reduced, it does not efficiently participate in the thiazole-forming reaction. In contrast, the inclusion of the presumed natural reductant (NADPH, FldA, and Fpr) resulted in

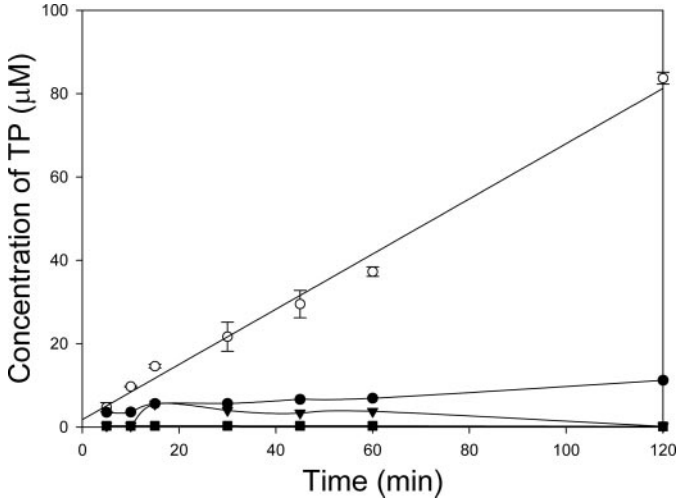

FIGURE 7. ***In vitro* time course of thiamine phosphate formation.** The TP formed in the assays was converted to the thiochrome, separated by HPLC, and quantified using a fluorescence detector. The conditions were as follows: complete assay (*open circles*), no Dxp (*closed circles*), no tyrosine (*triangles*), no AdoMet (*squares*). Data for the complete assay was fitted using Sigmaplot to a linear function with a rate of 0.66 ± 0.02 μM min^{-1}.

the observed Thz-P-forming activity and was used for all subsequent experiments where active ThiFS was required.

The rate of thiazole formation in the optimized *in vitro* assays was much slower than the relatively rapid reaction of tyrosine with the reduced AdoMet·ThiGH complex observed by EPR, suggesting that a step after the ThiH-dependent tyrosine cleavage may be rate-limiting for thiazole formation. Time courses in which tyrosine, Dxp, and AdoMet were each left out of the assay mixture showed that they were essential for activity (Fig. 7).

Identifying the Rate-limiting Protein Component—The observation that up to 1 eq of Thz-P was produced per ThiGH present in the assays was interesting, since either ThiGH or ThiFS could have been the limiting component, particularly since the system necessary to recycle ThiS after it has acted as a sulfur donor was not present in the assay mixture. This was tested by independently increasing the amounts of ThiGH or ThiFS present in the assay and quantifying the Thz-P after conversion to the thiochrome. The standard assay mixture included 65 μM ThiGH and 96 μM ThiFS and gave a rate of Thz-P formation of 25 μM/h. Keeping all other components constant but increasing the amount of ThiGH from 65 to 130 μM increased the rate from 25 to 58 μM/h, but increasing the concentration of ThiFS from 96 to 191 μM gave an almost unchanged rate of reaction (24 μM/h). From these data, it is clear that the ThiGH in the assays is the rate-limiting protein component and seemed to achieve at most a single turnover under these conditions. Several lines of reasoning can be considered to explain these observations. Thiamine biosynthesis in *E. coli* is known to be tightly regulated (38), and biosynthesis is likely to be controlled by more than one mechanism. The expression levels of biosynthetic proteins from the *thiCEFSGH* operon are controlled, at least in part, by a "riboswitch" mechanism, although this recognizes the hydroxymethylpyrimidine portion of TPP and not the thiazole (41, 42). In addition, early studies with mutant strains of *S. typhimurium* have identified

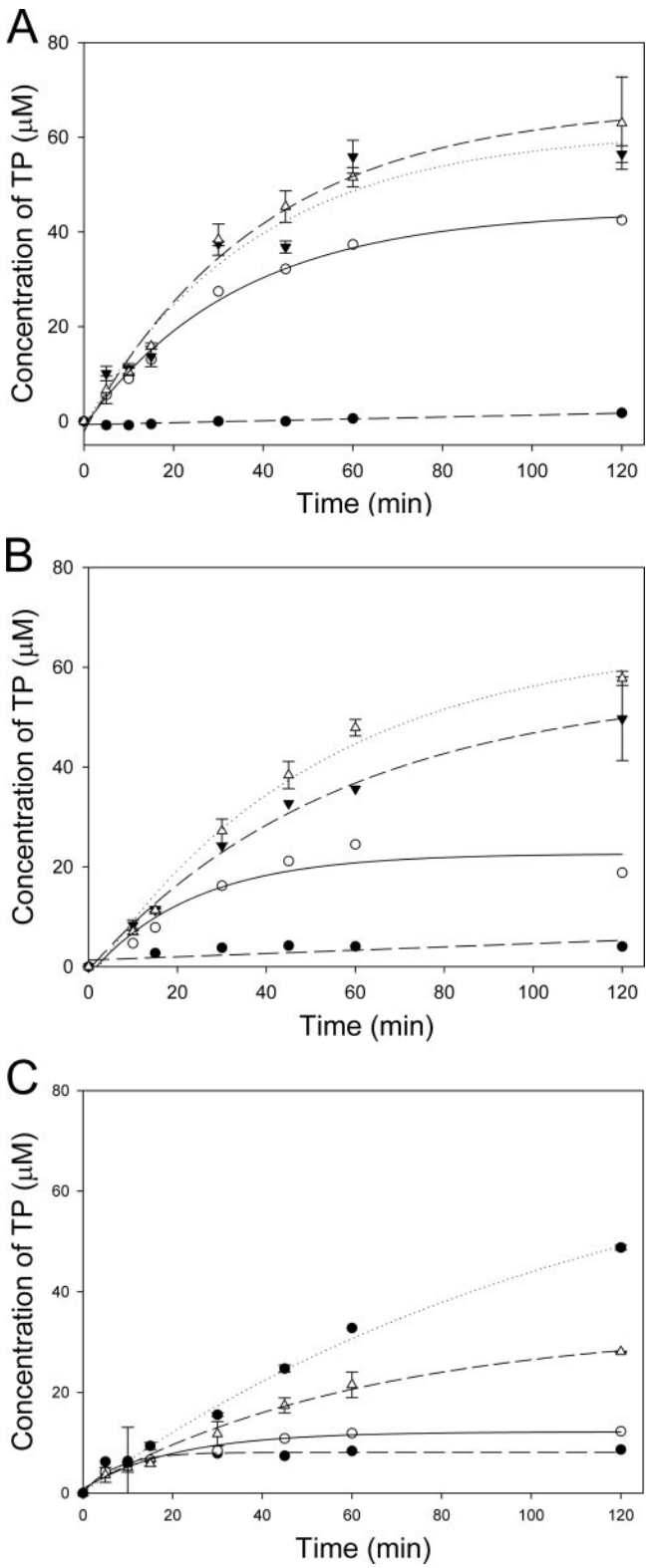

FIGURE 8. **Dependence of thiamine phosphate formation on ThiGH substrates.** Each *panel* shows the time dependence of thiamine formation on different substrates at a range of concentrations. *A*, effect of tyrosine concentration on TP formation. Tyrosine was present at the following concentrations: 0.0 mM (●), 0.1 mM (○), 0.3 mM (▼), and 0.5 mM (△). *B*, effect of AdoMet concentration on TP formation. AdoMet was present at the following concentrations: 0.0 mM (●), 0.1 mM (○), 0.3 mM (▼), and 0.5 mM (△). *C*, effect of Dxp concentration on TP formation. Dxp was present at the following concentrations: 0.0 mM (●), 0.1 mM (○), 0.3 mM (▼), and 1.0 mM (△). Curves were fitted using Sigmaplot, and the kinetic parameters and goodness of fit are shown in Table 3.

TABLE 3

Kinetic parameters for the dependence of thiamine phosphate formation on ThiGH substrates

Data were derived from Fig. 8. Data were fitted to an equation of the form, $y = A(1 - \exp(-Bx))$, where y is the concentration of TP formed, x is the concentration of the substrate, A is the calculated final concentration of TP formed, and B is the apparent first order rate constant. Results are shown with S.E., and R^2 is a measure of the goodness of fit.

Substrate	Concentration of substrate	Final concentration of TP (A)	Rate constant (B)	R^2
	mM	μM	$\times 10^{-2}$ min^{-1}	
Tyrosine	0.0[a]			
	0.1	45 ± 1.9	97 ± 0.1	0.99
	0.3	62 ± 5.2	98 ± 0.4	0.97
	0.5	80 ± 4.8	98 ± 0.2	0.99
AdoMet	0.0	4.5 ± 0.9	96 ± 2.2	0.80
	0.1	23 ± 2.8	96 ± 1.3	0.91
	0.3	71 ± 5.5	99 ± 0.2	0.99
	0.5	65 ± 6.3	98 ± 0.4	0.98
Dxp	0.0	8.1 ± 0.2	13 ± 1.6	0.98
	0.1	12 ± 0.6	6.0 ± 0.9	0.98
	0.3	37 ± 2.6	1.2 ± 0.1	0.99
	1.0	73 ± 9.7	0.9 ± 0.2	0.99

[a] Data for samples with no tyrosine resulted in very low concentrations of TP and were fitted to a straight line.

another system for controlling Thz-P biosynthesis and indicate that it does not operate at the transcriptional or translational level (43), although the precise mechanism has yet to be characterized. The *in vitro* assays for thiazole formation contained an excess of the reagents and enzymes required to convert Thz-P to TP, thus ensuring that this step was never rate-limiting (and control experiments showed that this was the case). It is therefore unlikely that sufficient Thz-P was accumulating to act as a negative regulator of ThiGH activity. ThiH shows sequence similarity to other members of the radical-AdoMet family (44), including lipoyl and biotin synthase that are required for the formation of the cofactor lipoic acid and the vitamin biotin, respectively. Experiments with these related proteins indicate that they carry out only a single turnover *in vitro* (45, 46), although the sulfur required for LipA and BioB is probably derived from a protein-bound iron sulfur cluster, which may be one of the mechanisms for limiting their *in vitro* activity. Since the immediate sulfur source for the thiazole is ThiS thiocarboxylate (or its acyl persulfide with ThiF), such a direct mechanism of inactivation upon turnover would not be expected for ThiGH. Finally and perhaps most likely, it may be that ThiGH is sensitive to product inhibition by a molecule other than Thz-P (*e.g. in vitro* experiments with biotin synthase have shown it to be inhibited by 5′-deoxyadenosine, the product of the reductive cleavage of AdoMet (47)).

Substrate Dependence of TP Formation—Since the Thz-P-forming reaction was not catalytic, it was not appropriate to determine Michaelis constants for the substrates. However, the dependence of the yield of TP on tyrosine, AdoMet, and Dxp was studied in more detail by varying their concentration and measuring the TP formation over time (Fig. 8, Table 3). No Thz-P was formed in the absence of tyrosine, and tyrosine became limiting at a slightly substoichiometric concentration (100 μM), but at higher concentrations (above 200 μM), tyrosine was not limiting, yielding ~55 μM TP. The reaction showed an absolute requirement for AdoMet, and in the absence of AdoMet, Thz-P formation was not observed. The maximum amount of Thz-P was obtained in assays with at least 300 μM

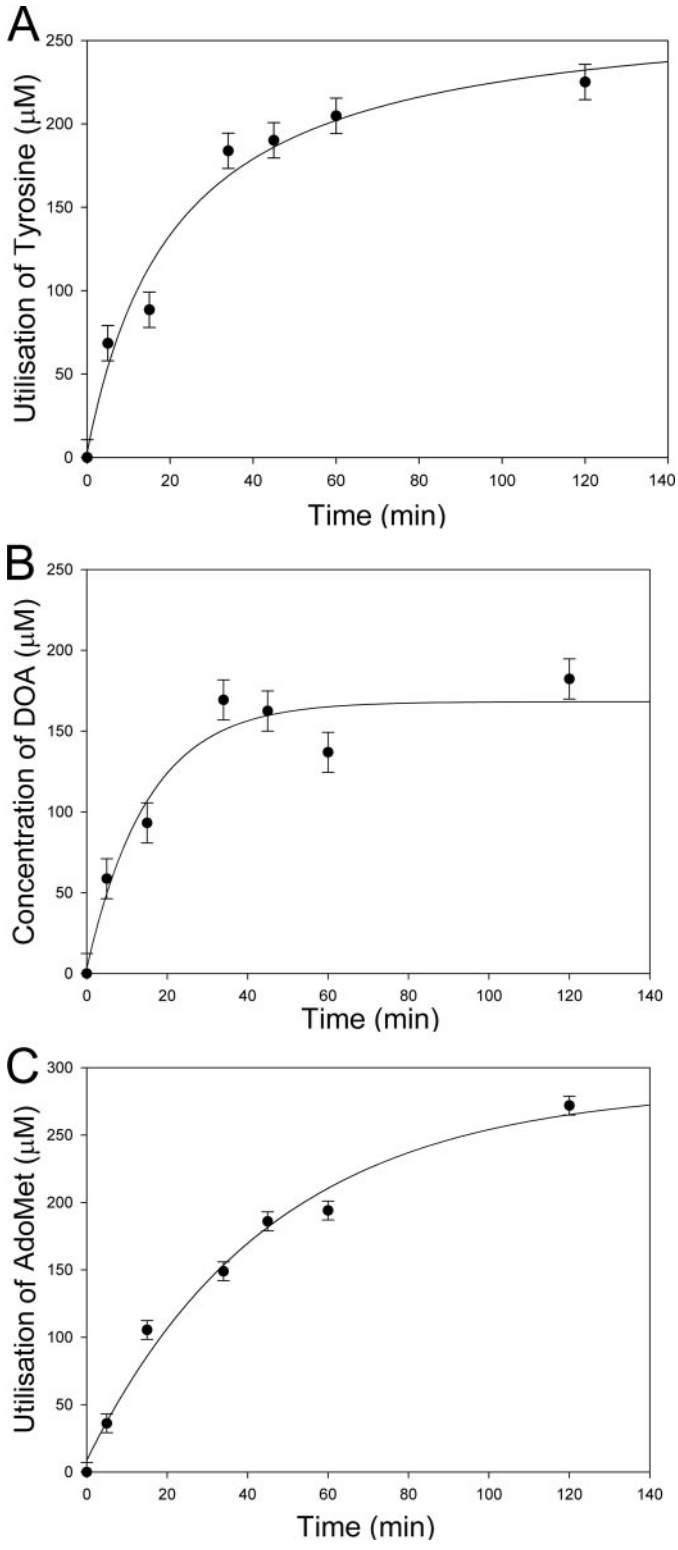

FIGURE 9. **LCMS analysis of a ThiGH assay.** The changes in concentrations of three components were monitored from each assay. *A*, utilization of tyrosine; *B*, formation of 5′-deoxyadenosine; *C*, utilization of AdoMet. Curves were fitted using Sigmaplot, and the kinetic parameters and goodness of fit are shown in Table 4.

AdoMet. The reaction showed a similar absolute requirement for Dxp, and the amount of Thz-P formed in the assay increased with Dxp concentration over the range of 0–1000 μM tested. The highest concentration of Thz-P formed was 47 μM at a Dxp

TABLE 4

Kinetic parameters for the time course of a ThiH assay

Data were derived from Fig. 9. Data were fitted to an equation of the form, $y = A(1 - \exp(-Bt))$, where y is the change in the concentration of a component (tyrosine, DOA, or AdoMet), t is time, A is the calculated final change in concentration, and B is the apparent first order rate constant (min^{-1}). Results are shown with S.E., and R^2 is a measure of the goodness of fit.

Substrate/Product	Final change in concentration (A)	Rate constant (B)	R^2
	mM	$\times 10^{-2}\ min^{-1}$	
Utilization of tyrosine	0.23 ± 0.02	4.4 ± 0.9	0.97
Formation of DOA	0.17 ± 0.01	6.8 ± 2.1	0.94
Utilization of AdoMet	0.28 ± 0.02	2.4 ± 0.4	0.98

concentration of 1.0 mM, which was in agreement with the amount of Thz-P observed in the other assays. In these assays, the amount of thiazole formed is therefore ~0.5 mol eq relative to ThiGH, which is at the lower end of the observed range (0.5–1 mol eq), and this variability probably reflects the difficulty of reconstituting the [4Fe-4S] cluster.

HPLC-MS Analysis of ThiGH Turnover—The proposed mechanism of the reaction involving ThiGH requires a 1:1 stoichiometry of the substrates AdoMet and tyrosine, and these would be expected to produce 1 mol eq of DOA from each reaction. By comparison with standards of known concentrations, HPLC-MS was used to measure the quantity of tyrosine and AdoMet utilized and the amount of DOA produced during a time course experiment. The results are shown in Fig. 9 and Table 4. In assays containing 255 μM ThiGH, the amount of DOA formed over 2 h was 182 μM, and the ratio of DOA formed/tyrosine utilized was 0.8–0.87:1.0 throughout the time course. Taking into account the observed experimental error, it seems likely that the actual ratio from the turnover of ThiGH is 1:1. The apparent utilization of AdoMet was higher, 272 μM over 2 h, but the instability of AdoMet probably explains this difference, and a species with a mass corresponding to adenine, a known product of AdoMet degradation (39), was observed to accumulate throughout the time course.

CONCLUSION

E. coli ThiGH has been reconstituted to contain a [4Fe-4S] cluster under anaerobic conditions. EPR spectra of ThiGH were consistent with near stoichiometric binding of AdoMet to the [4Fe-4S] cluster, which was then much more labile to reduction to the $[4Fe\text{-}4S]^{+1}$ state. An *in vitro* system containing purified *E. coli* proteins, ThiGH and ThiFS, together with tyrosine, Dxp, AdoMet, and a suitable reductant was able to form up to 1 mol eq of Thz-P. This limitation to 1 mol of product has also been observed for other proteins from the radical-AdoMet family. The results are consistent with the mechanism of thiazole formation proposed for facultative and obligate anaerobes, which make use of a radical-mediated cleavage of tyrosine to yield the dehydroglycine intermediate required by ThiG for the formation of Thz-P.

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Marco Kriek, Filipa Martins, Roberta Leonardi, Shirley A. Fairhurst, David J. Lowe and Peter L. Roach

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