Biosynthesis of the Thiazole Moiety of Thiamin Pyrophosphate (Vitamin B1)†

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ABSTRACT: While most of the proteins required for the biosynthesis of thiamin pyrophosphate have been known for more than a decade, the reconstitution of this biosynthesis in a defined biochemical system has been difficult due to the novelty of the chemistry involved. Here we demonstrate the first successful enzymatic synthesis of the thiazole moiety of thiamin from glycine, cysteine, and deoxy-D-xylulose-5-phosphate using overexpressed *Bacillus subtilis* ThiF, ThiS, ThiO, ThiG, and a NifS-like protein. This has facilitated the identification of the biochemical function of each of the proteins involved: ThiF catalyzes the adenylation of ThiS; NifS catalyzes the transfer of sulfur from cysteine to the acyl adenylate of ThiS; ThiO catalyzes the oxidation of glycine to the corresponding imine; and ThiG catalyzes the formation of the thiazole phosphate ring. The complex oxidative cyclization reaction involved in the biosynthesis of the thiamin thiazole has been greatly simplified by replacing ThiF, ThiS, ThiO, and NifS with defined biosynthetic intermediates in a reaction where ThiG is the only required enzyme.

Thiamin pyrophosphate is an essential cofactor in all living systems where it functions to stabilize the acyl carbanion synthon (*1*). The biosynthesis of this cofactor in *Escherichia coli* is outlined in Figure 1. The 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (HMP-PP)[1] is formed from 5-aminoimidazole ribonucleotide (AIR) in a reaction involving a complex, poorly understood rearrangement; the 4-methyl-5-(β-hydroxyethyl) thiazole phosphate (Thz-P) is formed from tyrosine, 1-deoxy-D-xylulose-5-phosphate (DXP), and cysteine; the thiazole and the pyrimidine are then coupled to give thiamin monophosphate (TMP), and a final phosphorylation gives thiamin pyrophosphate (TPP), the biologically active form of the cofactor (*2*−*5*).

The formation of the thiazole moiety of thiamin is complex and requires six gene products: ThiS, ThiF, ThiG, ThiH (*6*), IscS (*7*), and ThiI (*8*, *9*). The early steps in the sulfur incorporation chemistry have been worked out, and a novel acyl disulfide intermediate, covalently linking ThiF and ThiS, has been identified (*10*). However, despite the identification of an advanced intermediate, we have not been successful in reconstituting the thiazole biosynthesis using the *E. coli* proteins, possibly due to problems overexpressing and isolating active ThiH, a putative iron sulfur cluster containing protein. This analysis was recently supported by the isolation of ThiH under anaerobic conditions (*11*).

In contrast to *E. coli*, *Bacillus subtilis* and many other bacteria do not use tyrosine or ThiH for the thiazole biosynthesis. Rather, these bacteria use glycine and ThiO, a stable flavoenzyme previously identified as a glycine oxidase (*12*−*14*). In this paper, we describe the successful reconstitution of thiazole biosynthesis in a defined biochemical system containing ThiO, ThiF, ThiS, ThiG, and a cysteine desulfurase, and the identification of the function of each of these proteins.

MATERIALS AND METHODS

[^{35}S]-cysteine (20−150 mCi/mmol) was from Amersham Biosciences, [1,2-^{13}C]-glycine was from Cambridge Isotope Laboratories (99% purity), and [1,2-^{14}C]-glycine (101 mCi/mmol) was from Amersham Biosciences. Thz-P, HMP, DXP, and [1-^{13}C]-DXP were synthesized as previously described (*15*−*17*).

Cloning of B. subtilis Thiazole Biosynthesis Genes. Standard methods were used for DNA restriction endonuclease digestion, ligation, and transformation (*18*). Plasmid DNA was purified with the Wizard Plus SV DNA miniprep kit (Promega). DNA fragments were separated by agarose gel electrophoresis, excised, and purified with the QiaQuick Gel Extraction kit (Qiagen). The plasmid pET-16b was obtained from Novagen. *E. coli* strain DH5α was used as a recipient for transformation during plasmid construction and for plasmid propagation and storage. A Perkin-Elmer GeneAmp PCR system 2400 and Platinum *Pfx* DNA polymerase (Gibco Life Technologies) were used for PCR. *B. subtilis* CU1065 genomic DNA was used as the template for PCR. Primer synthesis and DNA sequencing were carried out at the Cornell University Bioresource Center. Table 1 shows the plasmids constructed and used in this study.

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[1] Abbreviations: AIR: 5-aminoimidazole ribonucleotide; DXP: 1-deoxy-D-xylulose-5-phosphate (DXP); ESI-FTMS: electrospray ionization-Fourier transform mass spectrometry; HMP: 4-amino-5-hydroxymethyl-2-methylpyrimidine; HMP-P: 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate; HMP-PP: 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate; IPTG: isopropyl-β-D-thiogalactopyranoside; PLP: pyridoxal phosphate; TCA: trichloroacetic acid; ThiS-COAMP: the carboxy terminal acyl adenylate of ThiS; ThiS-COSH: the carboxy terminal thiocarboxylate of ThiS; Thz-P: 4-methyl-5-(β-hydroxyethyl) thiazole phosphate; TMP: thiamin monophosphate; TPP: thiamin pyrophosphate.

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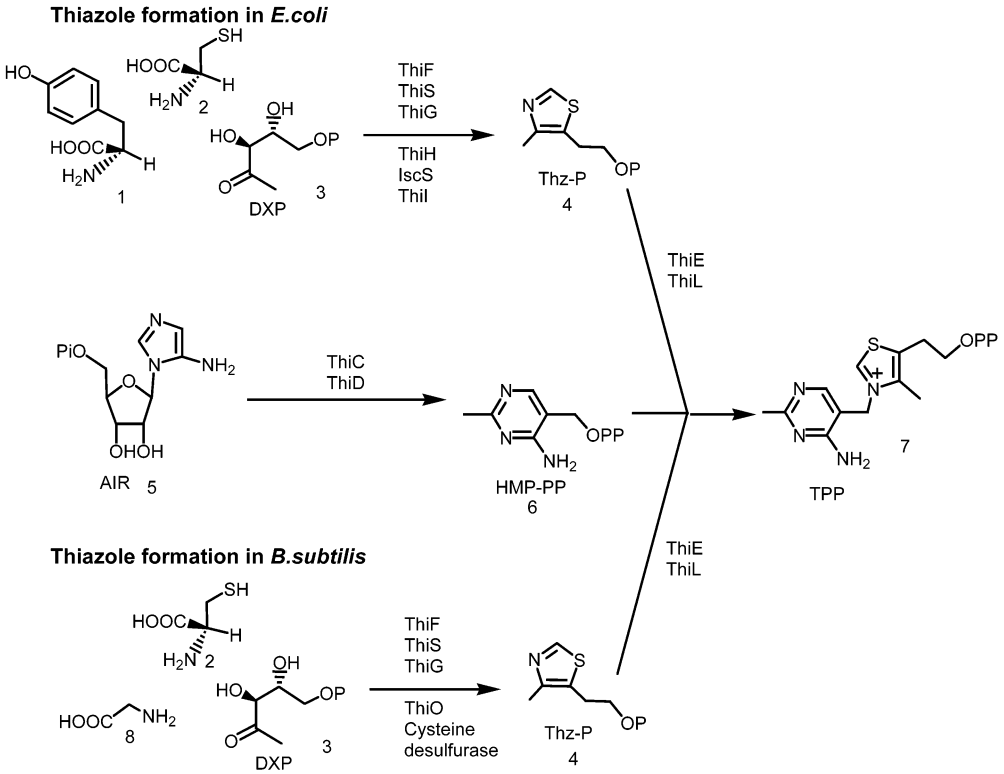

FIGURE 1: The bacterial thiamin biosynthetic pathways.

Table 1: Plasmids Constructed and Used in This Study

plasmids	*B. subtilis* gene	vector	ref
pCLK431	*thiF*	pET-16b	this study
pCLK820	*thiSG*	pET-16b	this study
pCLK811	*thiO*	pET-16b	9
pCLK421	*thiG*	pET-16b	this study
pCLK620	*thiD*	pET-16b	15
pCLK450	*thiE*	pET-16b	16
pTPB501[a]	*nifS*	pET-16b	this study
pTPB502[a]	*nifZ*	pET-16b	this study
pTPB503[a]	*yrvO*	pET-16b	this study
pTPB504[a]	*csd*	pET-16b	this study
pTPB503	*thiI*	pET-16b	this study

[a] Indicates that these genes were cloned with restriction enzymes, *Nde*I (5′) and *Bam*HI (3′). The others were with *Nde*I (5′) and *Xho*I (3′).

Overexpression of Proteins. E. coli BL21(DE3) containing the overexpression plasmid was grown in LB media containing ampicillin (50 μg/mL media) with shaking at 37 °C until its OD_{600} reached 0.5, at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 500 μM, and growth was continued for another 8 h at 25 °C. For the co-purification of ThiO, ThiSG, and ThiF, cultures containing pCLK811, pCLK820, and pCLK431 were prepared in 100, 200, and 100 mL of LB media, respectively, mixed, and harvested together. Cultures for HMP-P kinase (ThiD) and *B. subtilis* NifS-like proteins (NifS, NifZ, YrvO, and CSD) were prepared in 1 L of LB media. Thiamin phosphate synthase (*17*) was purified as previously described.

Purification of Proteins. The cells prepared above were resuspended in 5 mL of lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) and lysed by sonication (Heat Systems Ultrasonics model W-385 sonicator, 2 s cycle, 50% duty, 4 min). All proteins used in this study were purified using Ni−NTA resin following the manufacturer's instructions (Qiagen). After elution of the sample, protein solutions were desalted using a PD-10 column (Amersham, 50 mM Tris-HCl, pH 8.0).

Biosynthesis of Thz-P and Its Coupling with HMP-PP. A typical reaction mixture contained 100 μL of co-purified proteins (ThiOSGF, NifS, 160 μg), 10 mM glycine, 1 mM cysteine, 100 μM DXP, 1 mM HMP, 5 mM ATP, 5 mM $MgCl_2$, 10 μg of ThiD, 10 μg of ThiE, 20 μg of NifS and 50 μM PLP. The reaction mixtures were diluted to 150 μL with 50 mM Tris-HCl (pH 8), incubated at 25 °C for 1 h, and quenched with 150 μL of 10% (w/v) trichloroacetic acid.

In other reaction mixtures, NifS/PLP and cysteine were replaced by 1 mM Na_2S and 1 mM DTT. The reaction mixture using only ThiG for thiazole formation contained 100 μL of ThiG (60 μg), 10 mM glyoxylate, 10 mM ammonium chloride, 1 mM Na_2S, 1 mM DTT, 100 μM DXP, 1 mM HMP, 5 mM ATP, 5 mM $MgCl_2$, 10 μg of ThiD, and 10 μg of ThiE and was diluted to 150 μL with 50 mM Tris-HCl (pH 8).

Thiochrome Derivatization. Potassium acetate (50 μL of 4 M) was added to 100 μL of the quenched reaction mixture prepared above, followed by addition of 50 μL of potassium ferricyanide (30−40 mg/mL in 7 M NaOH). After 1 min, the reaction mixture was neutralized by the addition of 58 μL of 6 M HCl.

Detection of Thiochrome Phosphate by HPLC. Thiochrome phosphate was analyzed by HPLC (Hewlett-Packard) and detected by fluorescence (excitation at 365 nm and emission at 450 nm). A total of 100 μL of the thiochrome reaction mixture was injected onto a C_{18} column (Supelco, Supelcosil, LC-18-T 15 cm × 4.6 mm, 3 μm) and eluted using the following gradient: solvent A is water, solvent B is 0.1 M potassium phosphate, 4 mM tetrabutylammonium

hydrogen sulfate (pH 6.6), solvent C is MeOH, 0−3 min 10% B, 5% C, 3−20 min 5% B, 10% C, 20−24 min 5% B, 40% C, and for the column wash the following gradients were applied 24−26 min 40% B, 5% C, 26−29 min 40% B, 5% C and 29−40 min 10% B and 5% C. The flow rate was 1 mL/min. Commercial thiamin, thiamin monophosphate, and thiamin pyrophosphate were oxidized to the corresponding thiochromes and used as references eluting at 18, 20, and 22 min, respectively.

Alkaline Phosphatase Treatment. Biosynthesized thiochrome phosphate was purified by HPLC as described above. The eluted sample was lyophilized, redissolved in 500 μL of 50 mM Tris-HCl (pH 10.0), and treated with alkaline phosphatase (Sigma; 10 units for 12 h at 25 °C). The resulting reaction mixture was analyzed by HPLC for thiochrome as described above.

Analysis of Thz-P Formation by TLC/Autoradiography. 10 mM Na_2S, 200 μM DXP, and [1,2-^{14}C]-glycine (6.5 mM, 0.05 mCi) were incubated with ThiO (24 μg) and ThiG (20 μg) for 2 h in 100 μL of 50 mM Tris-HCl (pH 8.0). The reaction mixture was then applied to a TLC plate by repeated spotting with air-drying between applications. Thz-P (100 mM) was co-spotted allowing the visualization of Thz-P by UV (254 nm). After the final sample application, the TLC plate was dried and developed in a TLC chamber using 9:1:3 *N*-propanol/ethyl acetate/water. After the solvent had migrated ∼7 cm, the TLC plate was removed, dried, and exposed for 3 days to Kodak biomax MS autoradiography film using an intensifier screen (Kodak biomax transcreen − LE). The autoradiogram was developed in Kodak GBX developer, stopped, and fixed using GBX fixing solution.

TLC Purification and MS Analysis of Thz-P Biosynthesized from [1,2-^{13}C]-Glycine and [1-^{13}C]-DXP. Sample preparation and TLC development were as described above except that [1,2-^{13}C]-glycine and [1-^{13}C]-DXP were used. Nonlabeled Thz-P was spotted on each side of the reaction mixture for subsequent localization of [^{13}C]-Thz-P on the TLC plate. After development, the silica gel between the two reference spots was excised and transferred to an Amicon centrifugal concentrator (10 kDa membrane). The silica was then washed twice with 0.5 mL of MeOH/water (1:1), and the washings were partially evaporated before lyophilization. The resulting residue was dissolved in 20 μL of MeOH/water (1:1) and analyzed by ESI-MS (Negative ion mode, Esquire-LC 00146, 120 μL/h).

Preparation of ThiS, ThiS−COAMP, and ThiS−COSH. ThiSG and ThiF were separately purified from 400 mL cultures of the corresponding overexpression strains. For the preparation of ThiS−COAMP, a reaction mixture containing 150 μg of co-purified ThiSG, 50 μg of ThiF, 1 mM ATP, and 1 mM $MgCl_2$ in 100 μL of 50 mM Tris-HCl (pH 8) was incubated for 1 h at 25 °C. For the preparation of ThiS−COSH, a reaction mixture containing 150 μg of co-purified ThiSG, 50 μg of ThiF, 20 μg of NifS, 50 μM PLP, 1 mM cysteine, 1 mM ATP, and 1 mM $MgCl_2$ in 100 μL of 50 mM Tris-HCl (pH 8) was incubated for 1 h at 25 °C.

Characterization of ThiS, ThiS−COAMP, and ThiS−COSH by ESI−FTMS. All samples prepared as described above were desalted by loading onto a reverse-phase peptide trap (Michrom Bioresources, Auburn, CA). The trap was washed with 2 mL of 98:1:1 (H_2O/MeOH/HOAc), and eluted with 80 μL of 26:70:4 (H_2O/MeOH/HOAc). The solutions were electrosprayed at 1−50 nL/min with a nanospray emitter. Mass spectra were acquired on a 6 T modified Finnigan FTMS described previously (*19*). For MS/MS spectra, specific ions were isolated using stored waveform inverse Fourier transform (SWIFT) (*20*), followed by sustained off-resonance irradiation collisionally activated dissociation (CAD) (*21*). Assignment of the fragment masses and compositions were made with the computer program THRASH (*22*). After each mass value, the mass difference (in units of 1.00235 Da) between the most abundant isotopic peak and the monoisotopic peak is denoted in italics.

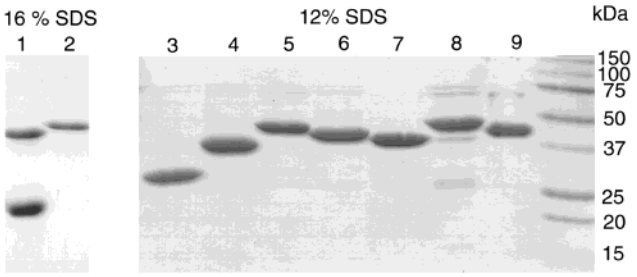

FIGURE 2: SDS−PAGE analysis of the purified thiazole biosynthetic proteins. Lane 1: Copurified ThiS and ThiG (from pCLK820), lane 2: ThiG (from pCLK421), lane 3: ThiG (from pCLK421), lane 4: ThiF (from pCLK431), lane 5: ThiO (from pCLK811), lane 6: NifZ (from pTPB502), lane 7: YrvO (from pTPB503), lane 8: CSD (from pTPB504), lane 9: NifS (from pTPB501). Lanes 1 and 2 were run on a 16% gel, and lanes 3−9 were run on a 12% gel. The molecular mass standards for the 12% gel are indicated on the right.

B. subtilis Cysteine Desulfurases as Sulfur Donors. 2 mM [^{35}S]-cysteine (20−100 μCi), 4 mM ATP, 10 mM $MgCl_2$, and ThiSGF (180 μg) were preincubated for 10 min before adding 3 μL of NifS (4.5 μg), NifZ (6.0 μg), CSD (10.2 μg), or YrvO (10.8 μg) to give a total volume of 50 μL (200 mM Tris-HCl pH 7.8). After 30 min, the small molecules were removed by desalting (BIO-RAD biospin 6, 10 mM Tris-HCl, pH 7.4, 0.02% sodium azide) directly into a microcentrifuge tube containing 50 μL of SDS−PAGE loading buffer. A total of 20 μL of each reaction mixture was analyzed by SDS−PAGE (15%). The developed gel was washed thoroughly with water and dried in vacuo between gel drying film (Promega) using a BIO-RAD gel dryer (model 583). The dried gel was exposed to biomax MR (Kodak) autoradiography film for 3 h to 3 days, developed using Kodak GBX developer and replenisher, washed with distilled water, fixed using Kodak GBX fixer and replenisher, and again thoroughly washed with distilled water before drying. The resulting autoradiogram was analyzed by densitometry (Epson expression 1600 with the software U-scan-IT-gel version 3.1) and/or scanned and analyzed using NIH image 1.62 freeware. The resulting intensities were used to calculate the relative efficiencies in intensity units/μmol of enzyme and the largest value was normalized to 100%.

Kinetics of Sulfur Transfer from Cysteine to ThiS. 2 mM [^{35}S]-cysteine (80−600 μCi), 4 mM ATP, 10 mM $MgCl_2$, and ThiSGF (720 μg) were preincubated for 10 min before adding 30 μL of YrvO (108 μg) in 200 mM Tris-HCl pH 7.8 (total volume 200 μL). Aliquots of the reaction mixture (20 μL) were quenched after 1, 7, 16, 38, 60, 96, 125, and 151 min by gel filtration (BIO-RAD biospin 6 in 10 mM

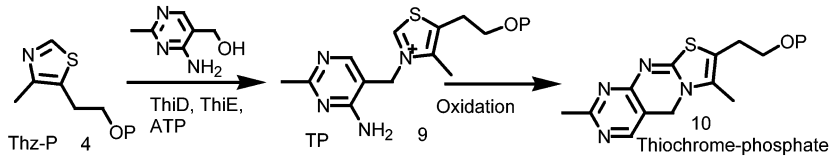

FIGURE 3: Assay procedure for the detection of thiazole phosphate biosynthesis.

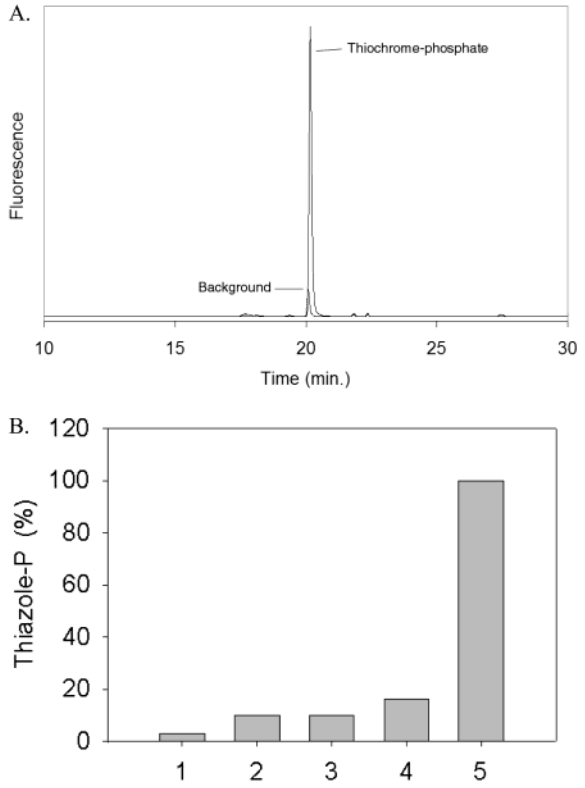

FIGURE 4: Reconstitution of thiazole phosphate biosynthesis. (A) HPLC analysis of the complete reconstitution reaction mixture containing ThiS, ThiG, ThiO, ThiF, NifS, ThiD, ThiE, DXP, glycine, ATP, $MgCl_2$, and cysteine. The major reaction product comigrated with an authentic sample of thiochrome phosphate. The background from the reaction mixture in which NifS, cysteine, glycine, and DXP were omitted is indicated. The residual thiamin phosphate comes from the thiamin phosphate that copurifies with ThiE (*17*). (B) Thiazole-phosphate biosynthesis in vitro is dependent on cysteine, DXP, and glycine. 1. Reconstitution reaction mixture without glycine, DXP, NifS, and cysteine. 2. Reconstitution reaction mixture without NifS and cysteine. 3. Reconstitution reaction mixture without glycine. 4. Reconstitution reaction mixture without DXP. 5. Complete reconstitution reaction mixture. The activity in column 5 was set to 100% and corresponded to the production of 0.75 nmol of thiazole phosphate in the reaction mixture.

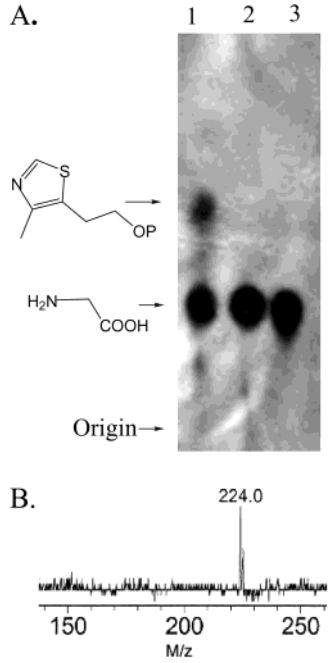

FIGURE 5: (A) Analysis of ^{14}C-glycine incorporation into Thz-P by autoradiography: 1. Reaction mixture containing ThiG, ThiO, DXP, sulfide, and [2-^{14}C]-glycine. 2. Reaction mixture without DXP. 3. Reaction mixture without proteins. The replacement of ThiF, ThiS, NifS, cysteine, and ATP with sulfide is discussed below. (B) ESI-FTMS (negative ion mode) of Thz-P biosynthesis using [1,2-^{13}C]-glycine and [1-^{13}C]-DXP. The molecular mass of unlabeled thiazole phosphate (Thz-OPO_3H^{-1}) is 222 Da. The glycine carboxylate carbon is lost as carbon dioxide.

Tris-HCl, pH 7.4, 0.02% sodium azide) directly into a microcentrifuge tube containing 20 μL of SDS−PAGE loading buffer. A total of 20 μL of each of the resulting samples was analyzed by SDS−PAGE (15%). The gel was developed and its autoradiogram was prepared as described above. The resulting autoradiogram was analyzed by densitometry. The resulting intensities were imported into Sigmaplot and fitted to an exponential function.

ThiS−COSH Is the Thiazole Sulfur Source. 2 mM [^{35}S]-cysteine (20−150 μCi), 4 mM ATP, 10 mM $MgCl_2$, and ThiSGF (180 μg) were preincubated for 10 min before adding NifS (6.2 μg) or YrvO (11.1 μg) in 200 mM Tris-HCl pH 7.8 (total volume 50 μL). After 1 h, the assay mixture was gel filtered (BIO-RAD biospin 6 in 10 mM Tris-HCl, pH 7.4, 0.02% sodium azide) into a microcentrifuge tube containing 20 μL of SDS−PAGE loading buffer. A total of 20 μL of this was analyzed by SDS−PAGE/autoradiography as described above to demonstrate successful formation of ThiS thiocarboxylate. The remaining sample (30 μL) was diluted to 120 μL with 200 mM Tris-HCl pH 7.7. A total of 30 μL of this sample (716000 CPM), was preincubated (20 min) with 3.6 mM HMP, 4 mM ATP, 10 mM $MgCl_2$, ThiD (60 μg), ThiE (12 μg), 8 mM glycine, 200 μM DXP, and the reaction was initiated by the addition of ThiG/ThiO (co-purified, 150 μg) to bring the total volume to 123 μL. Two control reactions were prepared in a similar fashion; one did not contain DXP, the other did not contain glycine. After 2.5 h at RT, 24 nmol thiamin-phosphate was added as carrier, and the reactions were quenched with 126 μL 10% TCA, and centrifuged at 4000*g* for 10 min to remove precipitated protein. The supernatant was centrifuged through a 10 kDa membrane and stored at −20 °C until use. Potassium acetate (25 μL, 4 M) and potassium ferricyanide (25 μL, 30 mg/mL in 7 M NaOH) were added to a 50 μL aliquot of this sample. After 1 min, the reaction was neutralized by the addition of 28 μL of 6 M HCl (pH must be 6.5−7.5). A total of 100 μL of the resulting solution was analyzed by HPLC (Detection: absorbance at 205, 254 nm, fluorescence excitation at 365 nm and emission at 450 nm, as well as in-line scintillation counting using a Packard flow scintillation analyzer 500TR

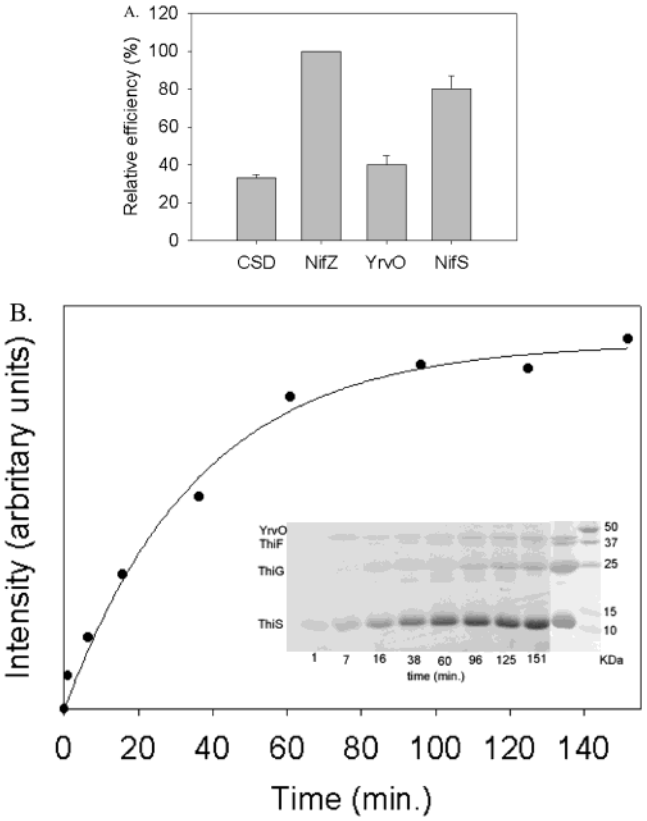

FIGURE 6: Efficiency of sulfur transfer from cysteine to ThiS catalyzed by the *B. subtilis* cysteine desulfurases. (A) Relative efficiency of sulfur transfer from cysteine to ThiS catalyzed by the *B. subtilis* cysteine desulfurases. The assay mixture contained [^{35}S]-cysteine, ATP, ThiG (co-purified with ThiS), ThiF, and one of the four cysteine desulfurases. (B) The rate of formation of ThiS thiocarboxylate catalyzed by YrvO obtained by autoradiography. The rate was determined by fitting the intensity obtained from the autoradiogram to a first-order function. Inset, SDS−PAGE (15%) and autoradiogram of the time-dependent formation of ThiS-thiocarboxylate by the cysteine desulfurase YrvO. The lane next to the molecular mass standards is a Coomassie stained gel of the proteins in the reaction mixture. The background labeling is likely due to sulfane sulfur, a byproduct of sulfur transferases, reacting with the cysteines on those proteins.

series.) The elution conditions were identical to those described above with a flow rate of 0.9 mL/min.

Reforming of ThiS from ThiS−COSH. ThiS−COSH was formed by incubating 2.5 mL of ThiSGF (5 mg/mL, freshly purified from 0.5 L of a ThiF overexpression culture and 1.0 L of a ThiSG overexpression culture) and 100 μL of purified ThiO (12 mg/mL) with cysteine (2 mM), ATP (4 mM), $MgCl_2$ (8 mM), and YrvO (0.5 mg). After ~95% conversion (determined by ESI-FTMS analysis, 1−5 h), the proteins were desalted (Amersham PD-10 desalting column in 25 mM Tris-HCl, pH 7.6). DXP (200 μM) or glycine (8 mM) was added to 500 μL of this sample and the conversion of ThiS−COSH to ThiS was monitored by ESI-FTMS analysis of the corresponding molecular ions.

RESULTS AND DISCUSSION

Overexpression of B. subtilis ThiO, ThiSG, ThiG, and ThiF. These were all overexpressed as soluble proteins in *E. coli* and purified by Ni−NTA affinity chromatography. ThiG was copurified with His-tagged ThiS for most reaction

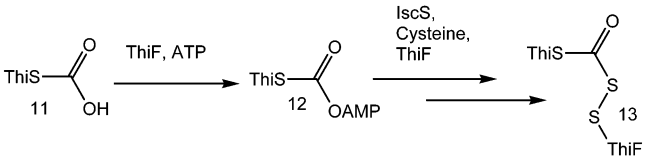

FIGURE 7: Formation of the ThiS−COS−S−ThiF complex in *E. coli*.

mixtures. The His-tags did not interfere with the activity (unpublished results).

Assay for Thiazole Phosphate. The assay strategy involved the enzymatic conversion of the thiazole phosphate (**4**) to thiamin phosphate (**9**), followed by oxidative cyclization to thiochrome phosphate (**10**), which can be readily analyzed by HPLC with fluorescence detection (Figure 3). A similar assay protocol has been previously used to detect low levels of thiazole biosynthesis in spinach chloroplast (*23*).

Reconstitution of Thz-P Biosynthesis using Purified B. subtilis ThiO, ThiF, ThiSG, and NifS. Glycine, DXP, and cysteine were incubated with purified ThiO, ThiSG, ThiF, and NifS, and the reaction mixture was analyzed for thiazole phosphate using the thiochrome assay (Figure 4A). We observed 1−5% conversion based on 100 μM DXP after 2 h. The level of thiazole phosphate synthesis dropped to near background levels when cysteine, DXP, or glycine was omitted (Figure 4B). The thiazole observed for experiments 2, 3, and 4 in Figure 4B may be due to protein bound intermediates or products.

Alkaline Phosphatase Treatment of the Biosynthesized Thiochrome Phosphate. To confirm the HPLC identification of thiochrome phosphate derived from biosynthesized thiazole phosphate, the putative thiochrome phosphate was dephosphorylated by treatment with alkaline phosphatase. The resulting product comigrated with authentic thiochrome by HPLC analysis.

Confirmation of Glycine and DXP as Thiazole Phosphate Precursors. When the reconstitution reaction was run in the presence of [1,2-^{14}C]-glycine and analyzed by TLC followed by autoradiography, the radioactivity comigrated with unlabeled Thz-P, which was visualized by UV (Figure 5A). In addition, when the reaction was run using [1,2-^{13}C]-glycine and [1-^{13}C]-DXP, analysis of the resulting thiazole-phosphate by ESI-MS demonstrated the production of Thz-P with an extra two mass units (Figure 5B). These results confirm that DXP and glycine are the precursors to the thiazole and demonstrate that we are observing de novo Thz-P biosynthesis in our reconstitution system. Glycine has been previously identified as a thiazole precursor in an in vivo labeling study (*24*).

Formation of ThiS−COSH using the B. subtilis cysteine desulfurases. Four cysteine desulfurases have been identified in *B. subtilis*: NifS (IscS ortholog), YrvO, NifZ, and CSD. Of these, NifS has been associated with nicotinamide biosynthesis and is an essential gene (*25*). CSD may be involved in iron sulfur cluster assembly, as it is adjacent to *yurV*, an IscU homologue. YrvO is localized adjacent to tRNA (5-methylamino)methyl-2-thiouridylate methyltransferase, and NifZ is adjacent to ThiI. All four cysteine desulfurases were analyzed for their efficiency in catalyzing the transfer of sulfur from cysteine to ThiS using SDS−PAGE and densitometry of the resulting autoradiogram (Figure 6A). These enzymes were also analyzed for their

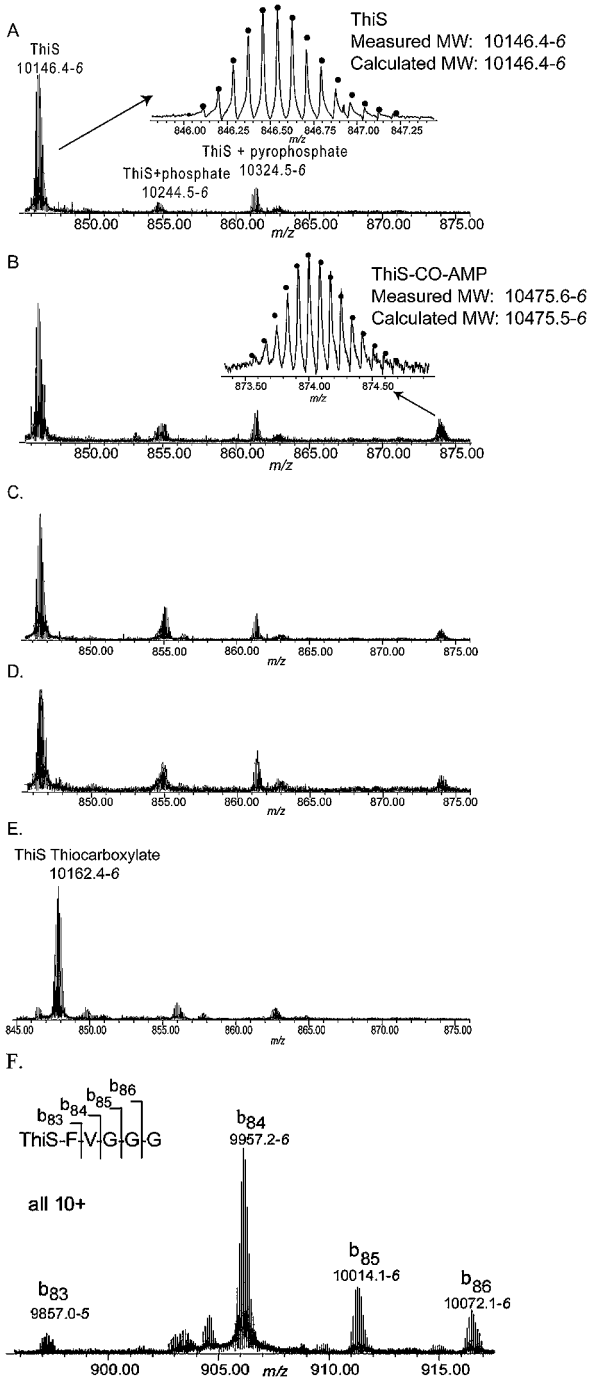

FIGURE 8: ESI-FTMS analysis of ThiS-thiocarboxylate formation. (A) Spectrum of ThiSG showing the ThiS signal. (B) Spectrum of ThiSG incubated with ATP, Mg^{2+}, and ThiF showing the formation of ThiS−CO-AMP. (C) Spectrum of ThiSG incubated with ThiF, ATP, Mg^{2+}, and cysteine, demonstrating that NifS is essential for the formation of ThiS thiocarboxylate. (D) Spectrum of ThiSG incubated with ThiF, ATP, Mg^{2+}, and NifS, demonstrating that cysteine is essential for the formation of ThiS thiocarboxylate. (E) Spectrum of ThiSG incubated with ThiF, ATP, Mg^{2+}, cysteine, DTT, and NifS showing the conversion of ThiS to ThiS thiocarboxylate. (F) Localization of the thiocarboxylate to the C-terminal end of ThiS. The figure shows the b ions resulting from SWIFT isolation and CAD activation of the ThiS thiocarboxylate peak. The marks made between the C terminal amino acids indicate the observed fragmentation pattern. The +16 Da modification is not observed in any of the fragments indicating that the +16 Da is on the C-terminal end. ThiS including the His-tag contains 87 amino acids. All ThiS clusters are from the 12+ charge state.

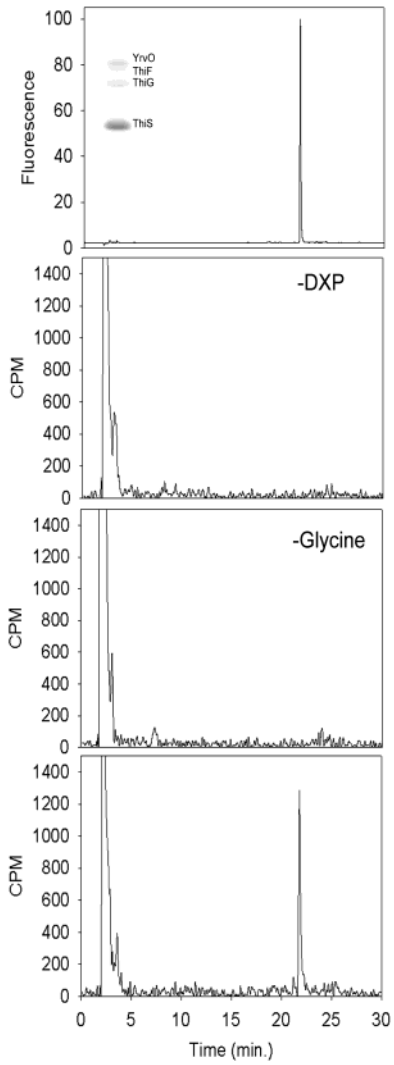

FIGURE 9: Demonstration that sulfur from ThiS thiocarboxylate is transferred to the thiazole. Top panel: HPLC analysis of a reference thiochrome phosphate sample. The inset shows the autoradiogram of the [^{35}S]-ThiS thiocarboxylate used for this experiment. Bottom panel: HPLC analysis of thiochrome generated from [^{35}S]-ThiS thiocarboxylate. The radiolabeled thiochrome was detected by in-line scintillation counting. Middle two panels: Control reactions from which DXP or glycine was omitted.

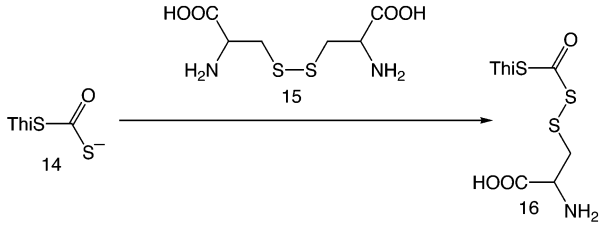

FIGURE 10: Trapping of ThiS-thiocarboxylate as the cysteine-acylpersulfide.

ability to form Thz-P using the thiochrome assay. NifZ was the most efficient sulfur transferase for ThiS−COSH formation, followed by NifS, YrvO, and CSD. YrvO, in our current reconstitution system, catalyzed the transfer of the sulfur from cysteine to ThiS at a rate of 0.027 min^{-1} (Figure 6B). NifZ was also the most efficient in forming thiazole-phosphate, but in both cases the differences are minimal (data not shown). Therefore, *B. subtilis* NifS, YrvO, NifZ, and CSD are all competent sulfur donors for the formation of ThiS-thiocarboxylate in vitro.

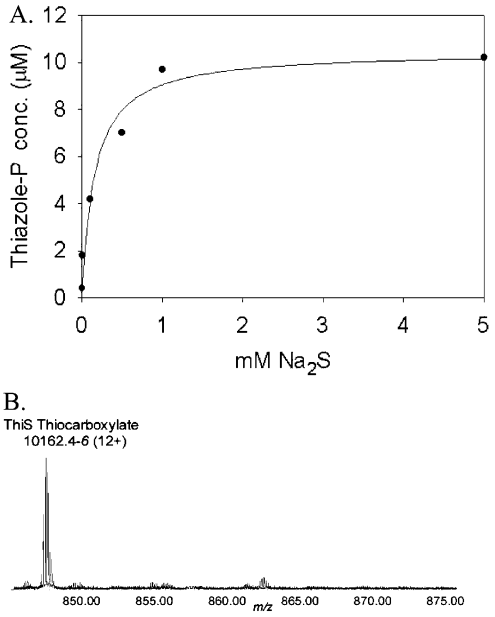

FIGURE 11: ThiS thiocarboxylate can be replaced by sulfide. (A) Sulfide concentration dependence of thiazole phosphate biosynthesis. The reaction mixture contained ThiO, ThiG, DXP, glycine, DTT, and varying concentrations of Na_2S (0, 0.01, 0.1, 0.5, 1, and 5 mM). (B) ESI-FTMS spectrum of ThiS thiocarboxylate formation using sulfide instead of NifS and cysteine.

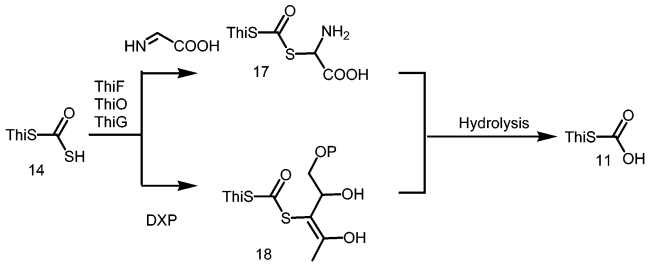

FIGURE 12: Two mechanistic proposals for the regeneration of ThiS from ThiS thiocarboxylate.

B. subtilis ThiI. ThiI is a required protein for the biosynthesis of thiazole phosphate as well as 4-thiouridine in *E. coli* (*8*). This protein is proposed to accept a sulfur from IscS-persulfide to give a ThiI-persulfide, which is subsequently used in the biosynthesis of thiazole phosphate and thiouridine. To determine if ThiI is required for thiazole formation in *B. subtilis*, the *B. subtilis* ortholog of ThiI was overexpressed and added to the reconstitution reaction mixture. We did not observe any enhancement in the rate of Thz-P formation. *B. subtilis* ThiI is missing the 120-amino acid carboxy terminal domain containing the proposed redox active cysteine residue in the *E. coli* enzyme; therefore, the possibility that active ThiI in *B. subtilis* is a two subunit enzyme cannot yet be excluded. It is also possible that the current reconstitution system is incomplete and therefore has a rate-limiting step that masks the effect of added ThiI.

Formation of ThiS Thiocarboxylate. In *E. coli*, ThiF catalyzes the formation of the acyl adenylate of ThiS (*10*), which then reacts with the IscS persulfide followed by a disulfide interchange to give a covalent ThiS−COS−S−ThiF complex (Figure 7). In contrast to the *E. coli* system, we do not detect a cross-linked ThiF/ThiS complex using the *B. subtilis* proteins.

Here we repeat the ThiS−COSH biosynthesis using *B. subtilis* ThiF/ThiS/NifS. ThiS was expressed and purified separately from ThiF to avoid the in vivo formation of ThiS−COSH; ESI-FTMS analysis of this sample confirmed its absence (Figure 8A). When ThiS was incubated with ThiF and ATP, the formation of ThiS−COAMP was observed by ESI-FTMS (Figure 8B). For the biosynthesis of ThiS−COSH, ThiS was incubated with ThiF, ATP, cysteine, and NifS. ESI-FTMS analysis of this reaction mixture demonstrated the formation of ThiS−COSH (Figure 8E,F) that is both NifS and cysteine dependent (Figure 8C,D).

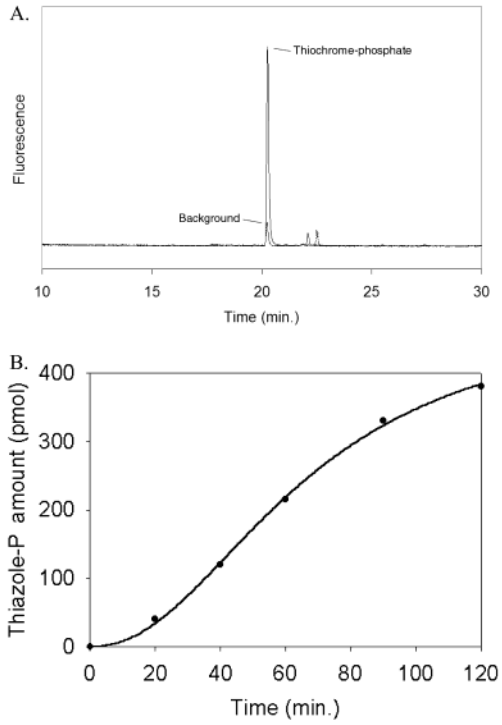

FIGURE 13: ThiG catalyzed thiazole phosphate formation: (A) HPLC trace of the reaction catalyzed by ThiG. (B) Time course for thiazole phosphate formation.

Thiazole Phosphate Biosynthesis using ThiS−COSH as the Sulfur Source. To confirm that ThiS−COSH is the sulfur source for Thz-P biosynthesis, we prepared [^{35}S]-ThiS thiocarboxylate using [^{35}S]-cysteine. The SDS−PAGE analysis of this reaction mixture verified the incorporation of the radioactivity from cysteine into ThiS (inset, Figure 9).

The radiolabeled ThiS−COSH was then desalted to remove the small molecules and used for thiazole phosphate biosynthesis. HPLC analysis of the reaction mixture demonstrated the formation of radiolabeled Thz-P (Figure 9, bottom panel, 9−15% conversion).To provide additional support for this, we trapped the sulfur on ThiS thiocarboxylate as the ThiS-cysteine acyl disulfide (**16**, Figure 10) by reacting ThiS thiocarboxylate with cystine (1 mM, 1 h). Under these conditions, the rate of thiazole formation decreased by a factor of 8 and the acyl persulfide could be detected by ESI-FTMS. This is the first direct demonstration that ThiS thiocarboxylate is the Thz-P sulfur source.

Replacement of ThiS−COSH with Na_2S. Since ThiS−COSH functions as a sulfide carrier, one can potentially regard this modified peptide as a source of sulfide. We therefore sought to determine if we could replace it with free sulfide in our reaction mixture. When 1 mM sulfide was used to replace NifS, ATP, cysteine, ThiS, and ThiF, we observed efficient formation of thiazole phosphate. The

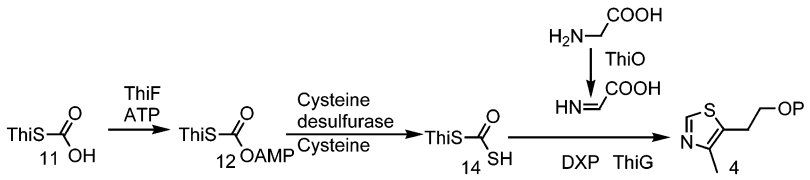

FIGURE 14: Functional assignment of ThiF, ThiS, ThiO, ThiG, and NifS in the biosynthesis of thiazole phosphate.

dependence of this reaction on sulfide concentration is shown in Figure 11. In addition, Na_2S was able to replace NifS and cysteine in the biosynthesis of ThiS thiocarboxylate; the ESI-FTMS spectrum of this reaction mixture is shown in Figure 11B. These experiments suggest that ThiS−COSH is simply a sophisticated source of sulfide, facilitating the transfer of sulfur from cysteine to an unidentified precursor to the thiazole phosphate.

The Hydrolysis of ThiS−COSH is DXP Dependent. We considered two possible mechanisms for the regeneration of ThiS from ThiS−COSH during thiazole formation (Figure 12). In the first mechanism, addition of ThiS−COSH to the glycine imine would give **17**. Hydrolysis of this intermediate or of an analogue of this intermediate would give ThiS (**11**). Alternatively, ThiS−COSH could add to DXP to give **18**, which could undergo hydrolysis to regenerate ThiS (**11**). The first mechanism predicts that ThiS regeneration will be dependent on the glycine imine, while the second proposal predicts that the regeneration of ThiS will be dependent on DXP.

To differentiate between these proposals, the conversion of ThiS−COSH to ThiS (in the presence of ThiG, ThiF, and ThiO) was monitored by ESI-FTMS after the addition of glycine or DXP. We observed that ThiS was reformed from ThiS−COSH after the addition of 200 μM DXP (60% conversion in 1 h, 100% conversion in 6 h), while 8 mM glycine had no effect. The hydrolysis of ThiS−COSH was >2000 times faster in the presence of DXP than in its absence (5% hydrolysis after 7 days).

ThiG Alone Can Catalyze the Formation of Thiazole Phosphate. We have previously demonstrated that ThiO is essential for thiazole phosphate biosynthesis (*12*) and catalyzes the oxidation of glycine to the glycine imine (*12*−*14*). This imine is unstable in aqueous buffer and is in equilibrium with glyoxylate and ammonia. This observation, combined with the demonstration here that cysteine, NifS, ThiF, and ThiS can be replaced with sulfide raises the possibility of a dramatic simplification of thiazole phosphate biosynthesis in which four of the five required proteins could be replaced with identified intermediates. To test this, a reaction mixture containing sulfide, ammonia, DXP, glyoxylate, and ThiG was incubated for 1 h at 25 °C and analyzed for thiazole phosphate production using the thiochrome assay. Thiazole phosphate is produced in approximately 70% of the yield found for the full system. The time course for this reaction is shown in Figure 13B and suggests that ThiG is the thiazole synthase.

SUMMARY

Here we demonstrate the first successful reconstitution of the thiazole moiety of thiamin in a defined biochemical system. This has enabled us to identify a biochemical function for each of the proteins involved: ThiF catalyzes the adenylation of ThiS, a cysteine desulfurase catalyzes the transfer of sulfur from cysteine to the acyl adenylate of ThiS, ThiO catalyzes the oxidation of glycine to the corresponding imine, and ThiG catalyzes the formation of the thiazole phosphate ring (Figure 14). We have been able to greatly simplify the thiazole phosphate formation, replacing ThiF, ThiS, ThiO, and NifS with defined biosynthetic intermediates in a reaction where ThiG is the only required enzyme. Mechanistic and structural studies to determined how ThiG catalyzes the complex process of thiazole phosphate formation are in progress.

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