

# A Missing Enzyme in Thiamin Thiazole Biosynthesis: Identification of TenI as a Thiazole Tautomerase<sup>‡</sup>

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 Supporting Information

**ABSTRACT:** In many bacteria *tenI* is found clustered with genes involved in thiamin thiazole biosynthesis. However, while TenI shows high sequence similarity with thiamin phosphate synthase, the purified protein has no thiamin phosphate synthase activity, and the role of this enzyme in thiamin biosynthesis remains unknown. In this contribution, we identify the function of TenI as a thiazole tautomerase, describe the structure of the enzyme complexed with its reaction product, identify the substrates phosphate and histidine 122 as the acid/base residues involved in catalysis, and propose a mechanism for the reaction. The identification of the function of TenI completes the identification of all of the enzymes needed for thiamin biosynthesis by the major bacterial pathway.

## INTRODUCTION

Thiamin is an important cofactor in all forms of life. It is biosynthesized in prokaryotes, lower eukaryotes and plants by a complex enzymatic pathway. The biosynthesis of thiamin in microorganisms has been studied extensively and most of the genes involved have been characterized.<sup>1,2</sup> The thiazole and the pyrimidine are biosynthesized by two separate unique mechanisms in bacteria and *Saccharomyces cerevisiae*. In *Bacillus subtilis*, the thiazole tautomer (*R,Z*)-2-(2-carboxy-4-methylthiazol-5(2*H*)-ylidene)ethyl phosphate (cThz\*-P) **14** is formed by an oxidative condensation of glycine, 1-deoxy-D-xylulose-5-phosphate (DXP) **1**, and cysteine,<sup>3–6</sup> and 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (HMP-PP) **17** is produced by a complex rearrangement of aminoimidazole ribonucleotide followed by phosphorylation.<sup>7–9</sup> Thiamin phosphate **18** is then formed by the coupling of the pyrimidine and the thiazole heterocycles (Figure 1a).<sup>9,10</sup> 2-(4-Methylthiazol-5-yl)ethyl phosphate (Thz-P) **15** and 2-(2-carboxy-4-methylthiazol-5-yl)ethyl phosphate (cThz-P) **16** are potential intermediates in this coupling reaction. A final phosphorylation gives thiamin pyrophosphate, the biologically active form of the cofactor.

In bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Geobacillus kaustophilus* and *Geobacillus stearothermophilus*, the thiaminase II gene (*tenA*) and the gene of unknown function (*tenI*) were found clustered with genes involved in thiamin thiazole biosynthesis<sup>8</sup> (Figure 1a in Supporting Information). Previous studies indicated the involvement of TenA (thiaminase II) and TenI (corresponding protein of unknown function) in the regulation of the production of extracellular enzymes like neutral protease, alkaline protease, and levansucrase.<sup>11</sup> However, since both TenA and TenI are strongly repressed by thiamin,<sup>12</sup> these regulatory roles did not provide any insights into the actual biochemical function of these two proteins. Subsequently, TenA was characterized as a thiaminase II involved in pyrimidine salvage from base-degraded thiamin.<sup>13,14</sup> The two genes do not

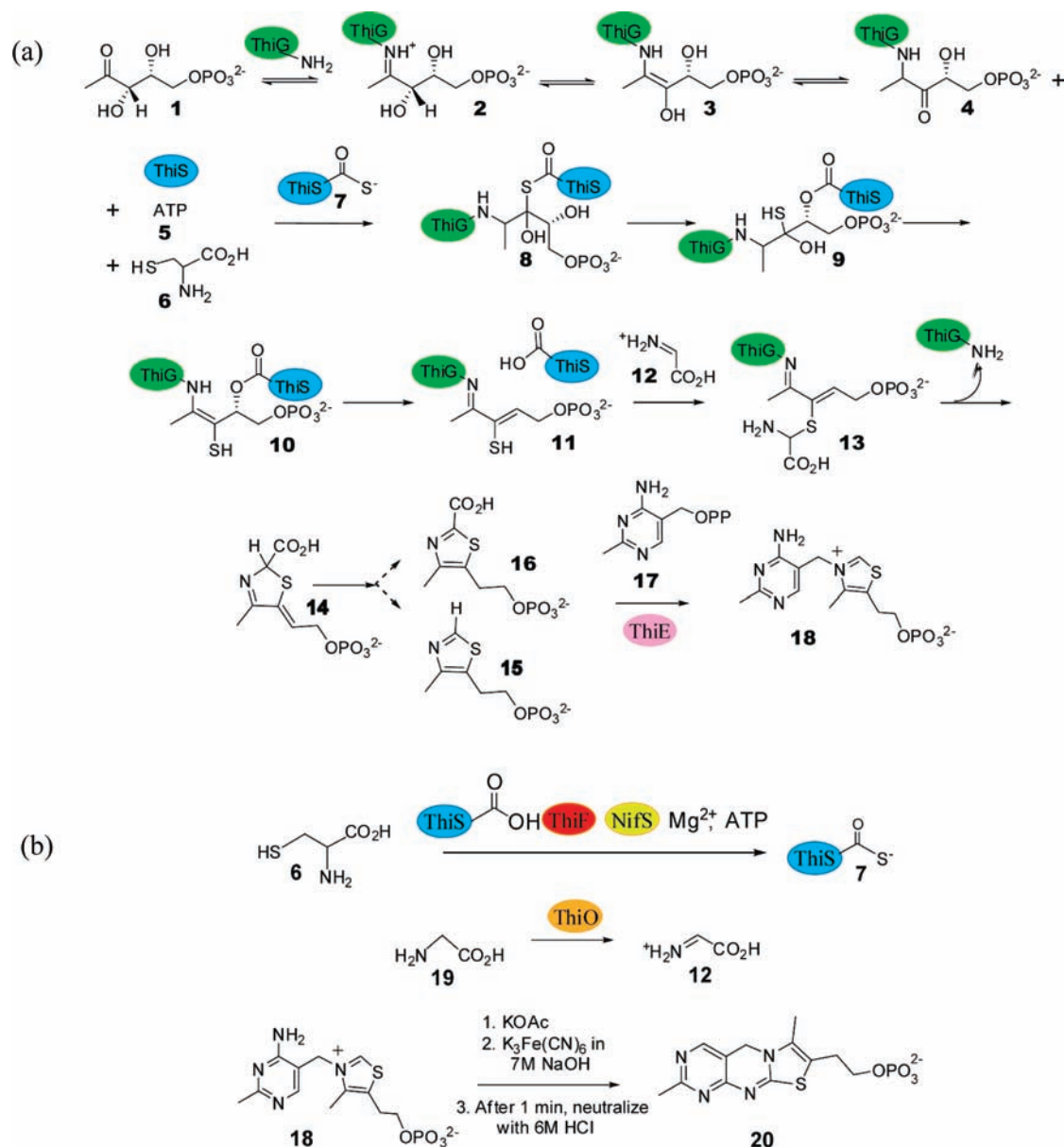
co-occur with high frequency, and TenI is less widely distributed than TenA.<sup>14,15</sup> *B. subtilis* TenI is a 22,783 Da protein,<sup>14</sup> and it shares high sequence similarity with thiamin phosphate synthase (ThiE) (Figure 2 in Supporting Information). Orthologues of TenI have been identified in bacilli, clostridia, members of the CFB (cytophagea/ flavobacter/ bacteriodes) group, *Campylobacter jejuni*, *Fusobacterium nucleatum*, *Chlorobium tepidum*, and *Aquifex aeolicus* and various other classes of bacteria and a ThiD–TenI–ThiE fusion has been found in *Porphyromonas gingivalis*. (Figure 1b in Supporting Information). We have previously reported a crystal structure of TenI, but the function of this protein in thiamin biosynthesis remained unknown.<sup>14</sup> In this paper, we identify the function of TenI as a thiazole tautomerase, describe the structure of the enzyme complexed with its reaction product, and propose a mechanism for the reaction.

## RESULTS AND DISCUSSION

**TenI Accelerates the Rate of Thiazole Formation.** To study the effect of TenI on the rate of in vitro thiazole formation, the reaction product was coupled with HMP-PP **17** and the rate of formation of thiamin phosphate **18** was measured. The thiazole reconstitution was accomplished by first incubating ThiF, NifS and ThiSG with cysteine and ATP for 1.5 h to form ThiS-COSH. Glycine, ThiO, HMP-PP, and ThiE were added, and the reaction was then initiated by adding DXP **1**. Aliquots of 50  $\mu$ L were quenched after 0, 1, 2, 5, 10, 20, 60, and 120 min. To quantitate the amount of thiamin phosphate **18** formed, each aliquot was oxidized to thiochrome phosphate, which was assayed by HPLC with fluorescence detection.<sup>3</sup> An identical reconstitution was run in the presence of TenI.

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**Figure 1.** (a) Mechanistic proposal for the formation of the thiamin thiazole in *B. subtilis*. The thiazole synthase (ThiG) reaction product **14** is coupled to HMP-PP **17** to form thiamin phosphate **18**. Thz-P **15** and cThz-P **16** are possible intermediates in this coupling reaction. (b) Reactions showing conversion of ThiS by ThiF and NifS into ThiS-COSH **7** (ThiS, ThiF, NifS, ThiG, ThiO are thiazole biosynthetic proteins), glycine oxidase (ThiO) converting glycine **19** to glycine imine **12**, and the oxidation of thiamin phosphate **18** to thiochrome **20**.

At short reaction times, TenI has a significant enhancement effect on the rate of thiazole formation. (Figure 2 and Supporting Information Figure 3). However, this effect is reduced at longer reaction times. This suggests that while TenI enhances the rate of thiazole formation, it is not essential for the process. The *in vitro* reconstitution reaction of thiazole biosynthesis is complex. Therefore, the next task was to identify which of the reaction components are enhanced by TenI.

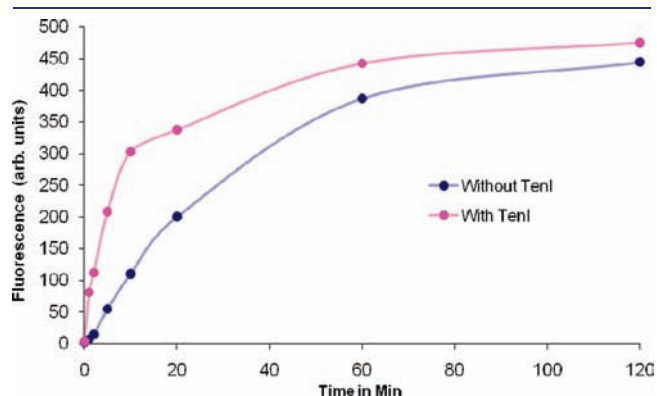
**TenI Does Not Affect Glycine Oxidase, Thiamin Phosphate Synthase, or ThiS-COSH Formation.** The glycine oxidase activity of ThiO was assayed in the presence and absence of TenI by monitoring hydrogen peroxide formation.<sup>16</sup> This experiment demonstrated that the rate of hydrogen peroxide production with increasing concentrations of glycine was unaffected by TenI (Figure 3a).

The involvement of TenI in the thiamin phosphate synthase (ThiE)-catalyzed coupling of Thz-P and HMP-PP was similarly probed. In each case thiamin phosphate was assayed by HPLC after oxidation to thiochrome phosphate.<sup>17</sup> TenI also has no effect on this reaction (Figure 3b).

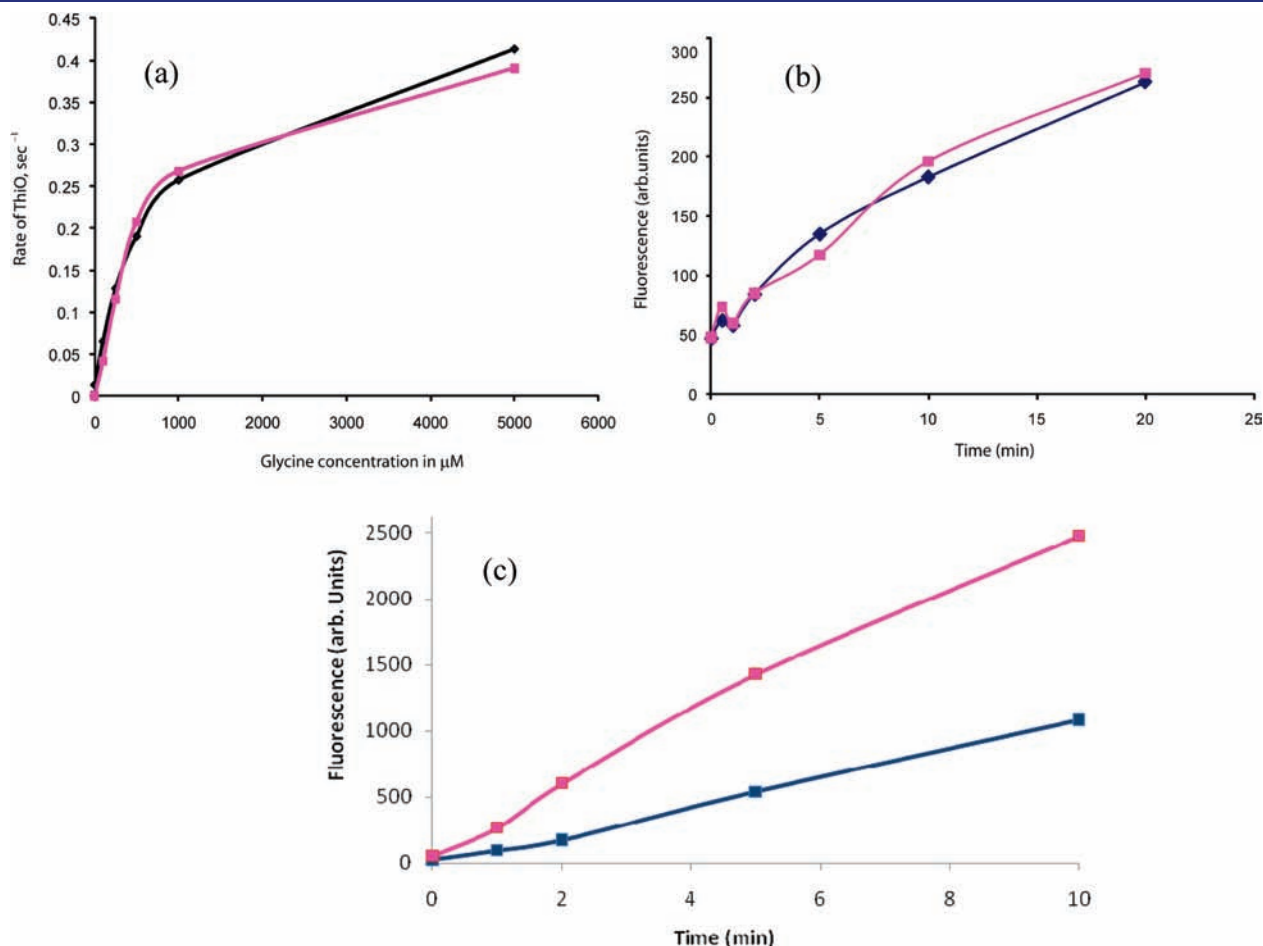
To evaluate the effect of TenI on ThiS-COSH formation, the thiazole reconstitution reaction was run using preformed ThiS-COSH in the presence and absence of TenI. If TenI was acting on ThiS-COSH formation, no TenI effect should be observed in this reconstitution. In the event, a clear increase in the rate of production of thiochrome could again be seen at the early stages of the reaction in the presence of TenI (Figure 3c). This suggests that TenI is not influencing the rate of ThiS-COSH formation and leaves only two possible functions for TenI: enhancement of the ThiG-catalyzed formation of cThz\*-P **14** or catalysis of the

conversion of cThz\*-P 14 to a better substrate for thiamin phosphate synthase.

Thz-P 15 is the characterized substrate for thiamin phosphate synthase and all of our mechanistic studies with this well-studied enzyme have been carried out using this compound.<sup>10,17,18</sup> The substrate tolerance of this enzyme now becomes important in the context of TenI function. In the reconstitution of the thiazole



**Figure 2.** Time course for the thiazole reconstitution reaction run in the presence and absence of TenI.



**Figure 3.** TenI does not affect glycine oxidase, thiamin phosphate synthase or ThiS-COSH formation. The blue trace is the reaction in the absence of TenI and the pink trace is the reaction in the presence of TenI. (a) The presence of TenI does not affect the rate of glycine oxidase (ThiO). (b) The presence of TenI does not affect the rate of the HMP-PP/Thz-P coupling reaction catalyzed by thiamin phosphate synthase. (c) The rate of the thiazole synthase-catalyzed reaction, using preformed ThiS-COSH, is enhanced by TenI.

biosynthesis described in Figure 1 (a), cThz\*-P 14, the product of the thiazole synthase,<sup>6</sup> is clearly a substrate for thiamin phosphate synthase. Therefore if TenI is catalyzing the conversion of cThz\*-P to a better substrate, Thz-P 15 and cThz-P 16 are the only possibilities (Figure 1). We therefore next evaluated cThz\*-P, cThz-P, and Thz-P as substrates for thiamin phosphate synthase.

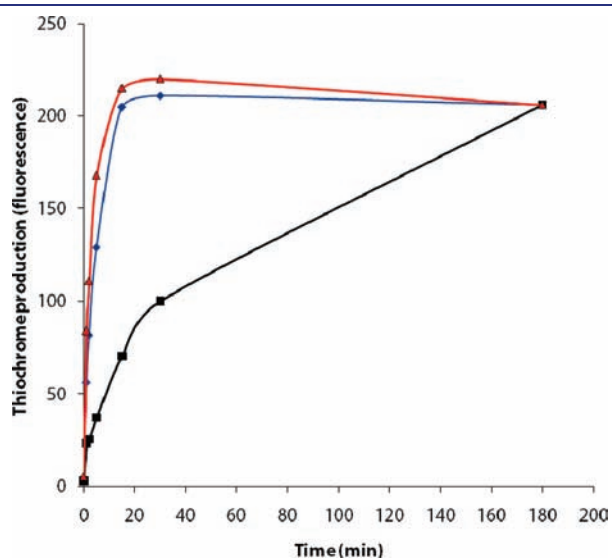
**Thiamin Phosphate Synthase (ThiE) Can Use Thz-P (15), cThz-P (16) and cThz\*-P (14) as Substrates.** The relative rates of the coupling of Thz-P, cThz-P, and cThz\*-P with HMP-PP 17 were measured. The reactions were quenched at 0, 1, 2, 5, 15, 30, and 180 min, and the thiamin phosphate formed was converted to thiochrome phosphate for HPLC analysis with fluorescence detection. The results are shown in Figure 4. Early in the course of the reaction, cThz-P and Thz-P are significantly better substrates than cThz\*-P. As expected from the data shown in Figure 2, the yield of thiamin phosphate is the same for all three substrates at longer reaction times. This experiment suggests that the enhanced formation of thiamin phosphate shown in Figures 2 and 4 may be a consequence of TenI-catalyzed aromatization of 14 to generate 15 or 16 which are better substrates for thiamin phosphate synthase. Previous experiments have conclusively shown the formation of thiamin phosphate by coupling of Thz-P 15 with HMP-PP.<sup>17,19</sup> Plausible routes by which cThz-P

16 and cThz\*-P 14 may be converted to thiamin phosphate are shown in Figure 5.

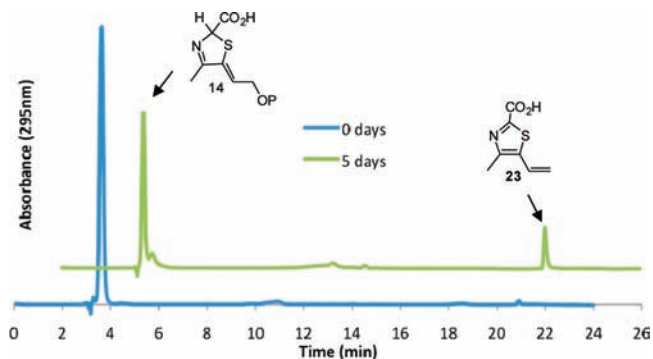
**Stability of cThz\*-P.** cThz\*-P is surprisingly stable and survives purification and structural analysis without undergoing aromatization.<sup>6</sup> On standing at room temperature, it slowly degrades to the carboxy vinylthiazole 23, with a half-life of approximately 13.5 days (Figure 6 and Supporting Information, Figure 6). As *B. subtilis* has a dividing time of about 30 min, in vivo aromatization of 14 clearly requires enzymatic catalysis.

**TenI Catalyzes the Aromatization of cThz\*-P to cThz-P.** Incubation of cThz\*-P 14 with TenI followed by HPLC analysis gave the chromatogram in Figure 7, showing the consumption of 14 (peak A) and the production of a new compound (peak B). Peak B was identified as cThz-P 16 (and not Thz-P 15) by comigration with authentic reference compounds.<sup>6</sup> It was not possible to carry out kinetic studies due to the scarcity of cThz\*-P which was prepared from a metabolite bound to the *S. cerevisiae* thiazole synthase (cThz\*-P preparation in Supporting Information).<sup>20,21</sup>

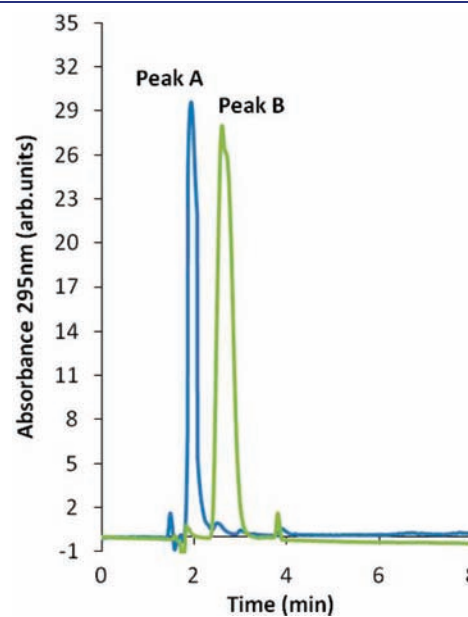
This conversion of cThz\*-P to cThz-P catalyzed by TenI was also verified by negative mode ESI-MS analysis of the reaction



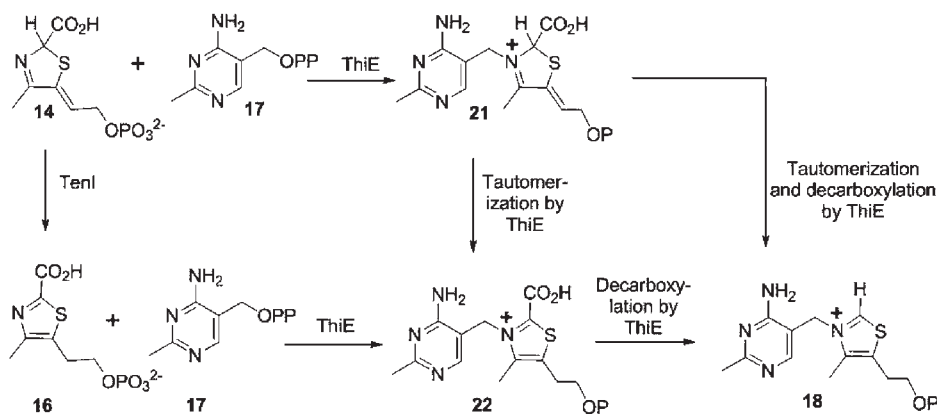
**Figure 4.** Substrate selectivity of thiamin phosphate synthase. The plot shows the time course for the alkylation of cThz\*-P 14 (black trace), Thz-P 15 (blue trace), and cThz-P 16 (red trace) by HMP-PP 17.



**Figure 6.** Reverse-phase HPLC analysis of the degradation of cThz\*-P 14 to carboxy vinylthiazole 23. The blue trace shows pure cThz\*-P, and the green trace shows the formation of the carboxy vinylthiazole as a degradation product after 5 days. Chromatograms are offset by 2 min.

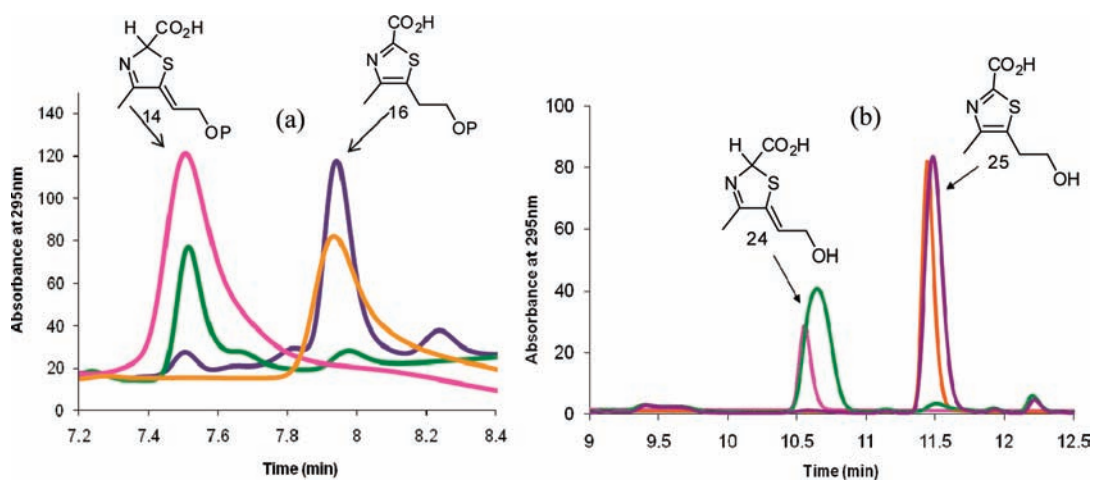


**Figure 7.** HPLC analysis demonstrating that TenI catalyzes the conversion of cThz\*-P 14 (blue trace, peak A) to a new compound (green trace) that comigrates with cThz-P 16 (peak B).

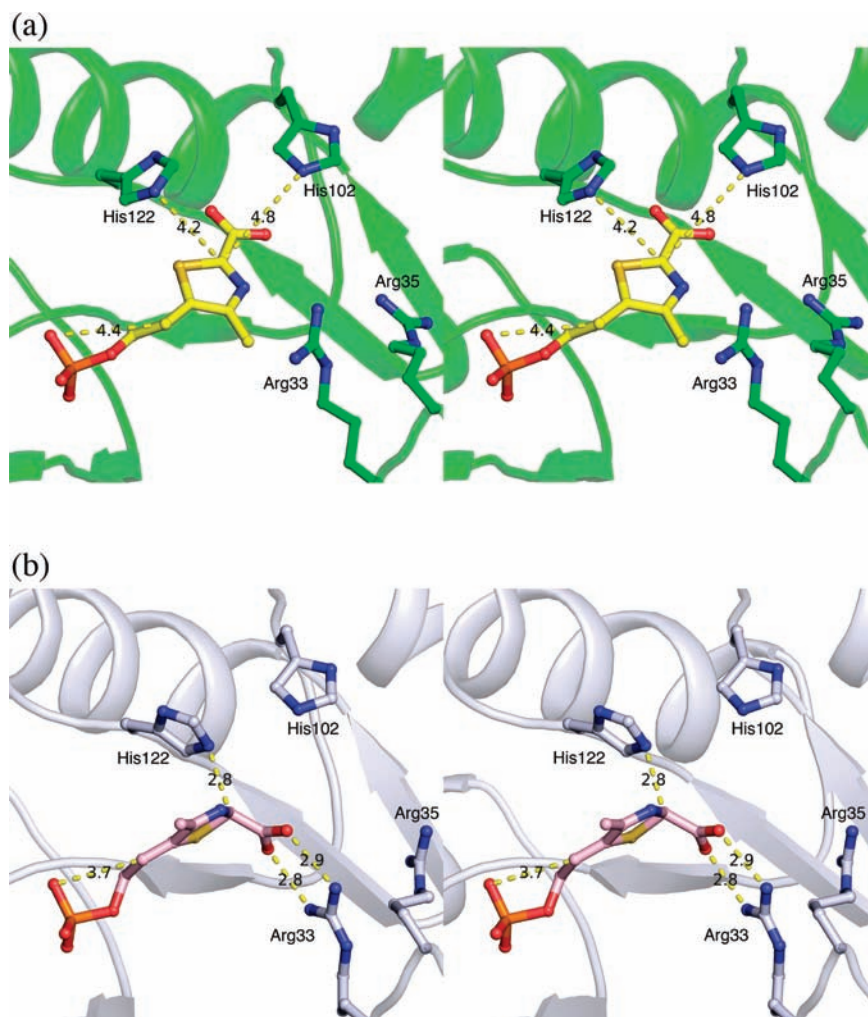


**Figure 5.** Routes for the conversion of cThz\*-P 14 and cThz-P 16 to thiamin phosphate 18. In bacteria lacking TenI, ThiE can catalyze the coupling of the thiazole tautomer 14 and HMP-PP 17 to give 21, which is converted to 18 directly or via intermediate 22.





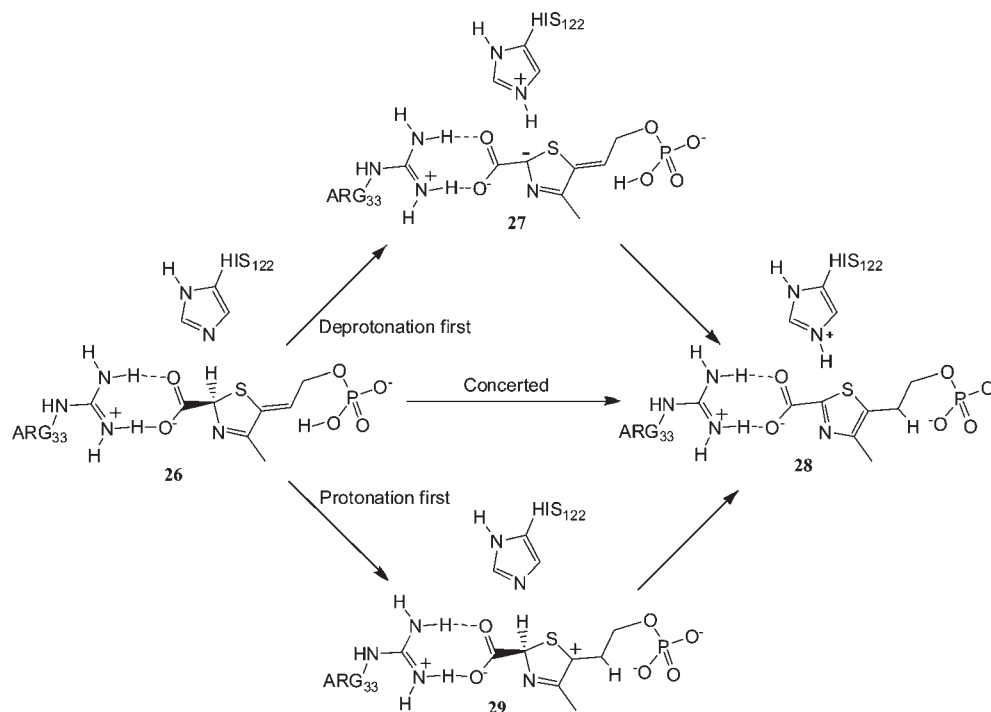
**Figure 8.** Reconstitution of thiazole biosynthesis in the presence and absence of TenI. (a) Comigration of the product of thiazole biosynthesis in the absence of TenI (green) with *cThz*\*-P (pink), and comigration of the product of thiazole biosynthesis in the presence of TenI (purple) with *cThz*-P (orange). (b) Comigration of the alkaline phosphatase-treated product of thiazole biosynthesis in the absence of TenI (green) with *cThz*\* (pink), and comigration of the alkaline phosphatase-treated product of thiazole biosynthesis in the presence of TenI (purple) with *cThz* (orange).



**Figure 9.** (a) Stereoview of the active site of the TenI with bound *cThz*-P. (b) Stereoview of a model of the active site of TenI with bound *cThz*\*-P.

(both *cThz*\*-P to *cThz*-P have distinct fragmentation patterns, Figure 7 in Supporting Information).

**Addition of TenI to the Thiazole Reconstitution Yields *cThz*-P.** The thiazole reconstitution reaction was run in the



**Figure 10.** Mechanistic proposals for the TenI-catalyzed thiazole aromatization reaction.

presence and absence of TenI as described above and analyzed by HPLC using a strong anion-exchange column (Figure 8a). In the absence of TenI, *cThz*<sup>\*</sup>-P was the only thiazole product formed, whereas in the presence of TenI, *cThz*-P was the major reaction product accompanied by a very small amount of *cThz*<sup>\*</sup>-P.

To further confirm the identity of the reaction product, *cThz*<sup>\*</sup>-P and *cThz*-P and the products of the two reconstitution reactions were dephosphorylated by treatment with alkaline phosphatase, and the resulting alcohols were reanalyzed by reverse phase HPLC. Again, the dephosphorylated product of the reconstitution reaction without TenI comigrated with (*R,Z*)-5-(2-hydroxyethylidene)-4-methyl-2,5-dihydrothiazole-2-carboxylic acid (*cThz*<sup>\*</sup>) 24, while the product of the reconstitution reaction run in the presence of TenI comigrated with 5-(2-hydroxyethyl)-4-methylthiazole-2-carboxylic acid (*cThz*) 25 (Figure 8b).

**Reversibility of the Aromatization Reaction Catalyzed by TenI.** Incubation of TenI with *cThz*-P did not result in the formation of detectable quantities of *cThz*<sup>\*</sup>-P by nuclear magnetic resonance (NMR) analysis. In addition, if TenI catalyzes the dearomatization of *cThz*-P, even at very low levels, it should be possible to observe TenI-dependent H/D exchange at C2 of the phosphoethyl side chain of *cThz*-P. In the event, incubation of *cThz*-P and TenI (1:1 ratio) in 50% D<sub>2</sub>O/H<sub>2</sub>O did not result in any detectable H/D exchange after 12 h. This is consistent with a large free energy difference between *cThz*-P and *cThz*<sup>\*</sup>-P.

**Structure of the TenI–*cThz*-P Complex.** To clarify the mechanism of the thiazole tautomerization reaction, a structure of TenI with the reaction product *cThz*-P 16 bound at the active site was obtained. The active-site structure of the enzyme is shown in Figure 9a. From this structure, it is not possible to unambiguously identify the acid and base required to catalyze the aromatization reaction. His102 and His122 are too far from the C2 of the thiazole and are improperly oriented for the deprotonation. In addition, a protein acid, to protonate C2' of the side

chain, is not apparent; the only proximal acid is the substrate phosphate group located 4.4 Å away. Assuming that this non-optimal arrangement of active-site residues was due to the substantial change in the structure of the substrate following aromatization, we modeled *cThz*<sup>\*</sup>-P into the active site. In this model (Figure 9b), His122 is reasonably positioned to deprotonate C2 of the thiazole tautomer, and the substrate phosphate is suitably positioned for protonation of the side-chain double bond. In addition, the substrate carboxylate forms hydrogen-bonding/electrostatic interactions with Arg33. In support of this model, the H122Q and H122A mutants are inactive (detection limit <0.5% wild-type activity), and *cThz*<sup>\*</sup> is not a substrate for TenI, indicating that the phosphate group is essential.

A mechanistic analysis of the thiazole aromatization reaction is outlined in Figure 10. Each reaction involves a phosphate-mediated protonation and a histidine-mediated deprotonation sequence. At this point, it is not possible to determine the order of the protonation/deprotonation reactions.

## CONCLUSIONS

*cThz*<sup>\*</sup>-P 14 is the product of the bacterial thiazole synthase. This thiazole tautomer is surprisingly stable, and its aromatization requires enzymatic catalysis. In this paper we demonstrate that TenI is the missing thiazole tautomerase and catalyzes an irreversible aromatization reaction converting *cThz*<sup>\*</sup>-P 14 to *cThz*-P 16. We also prove that, while thiamin phosphate synthase can convert each of *cThz*<sup>\*</sup>-P 14, *Thz*-P 15, and *cThz*-P 16 into thiamin phosphate, *cThz*-P 16 is likely the true substrate in vivo. A model for the structure of *cThz*<sup>\*</sup>-P 14 bound at the active site of TenI, based on the structure of the TenI/*cThz*-P complex, identifies the substrate phosphate and His122 as the acid/base residues involved in catalysis. The identification of the function of TenI completes the identification of all of the enzymes needed for thiamin biosynthesis by the major bacterial pathway.

## EXPERIMENTAL METHODS

**Source of Chemicals.** All chemicals and snake venom nucleotide pyrophosphatase were purchased from Sigma-Aldrich Corporation (U.S. A.) unless otherwise mentioned. Calf intestinal phosphatase was obtained from New England Biolabs. LB medium was obtained from EMD Biosciences. Kanamycin, ampicillin, and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) were purchased from LabScientific Inc. Nitrilotriacetic acid (NTA) resin was the NTA superflow by Qiagen. *E. coli* BL21(DE3) competent cells were obtained from Invitrogen. The microcon membrane filters were from Millipore. TALON metal affinity resin was purchased from BD Biosciences. Analytical HPLC (Agilent 1100 instrument) was carried out using a Phenomenex Gemini C18 110A (150 mm  $\times$  4.6 mm, 5  $\mu$ m ID) reverse phase column for thiochrome analysis, a Supelco LC-18-T (150 mm  $\times$  4.6 mm, 3  $\mu$ m ID) column for thiazole reconstitution analysis and a Phenosphere Strong Anion-Exchange 80A (250 mm  $\times$  4.6 mm, 5  $\mu$ m ID) column for the anion-exchange chromatography. HPLC purifications were carried out using a semiprep Supelco LC-18-T (250 mm  $\times$  10 mm, 5  $\mu$ m ID) column. HPLC grade solvents were obtained from Fisher Scientific. The Superdex 200 gel-filtration column was obtained from Pharmacia. A previously synthesized stock of [1- $^{13}$ C]-DXP<sup>22</sup> was used as the substrate of the thiazole reconstitution reactions and previously synthesized cThz-P and cThz<sup>6</sup> were used as reference compounds for the TenI-catalyzed reaction.

**Enzyme Overexpression and Purification.** *E. coli* BL21(DE3) containing the ThiSG overexpression plasmid (ThiG is copurified with ThiS for stability) in pET16b was grown in LB medium containing ampicillin (40  $\mu$ g/mL) with shaking at 37  $^{\circ}$ C until the OD<sub>600</sub> reached 0.6. At this point, protein overexpression was induced with IPTG (final concentration = 2 mM), and cell growth was continued at 15  $^{\circ}$ C for 16 h. The cells were harvested by centrifugation, and the resulting cell pellets were stored at  $-80$   $^{\circ}$ C. To purify the protein, the cell pellets from 1 L of culture were resuspended in 25 mL of lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8) and lysed by sonication (Heat Systems Ultrasonics model W-385 sonicator, 2 s cycle, 50% duty). The resulting cell lysate was clarified by centrifugation and the ThiSG protein was purified on Ni-NTA resin following the manufacturer's instructions. After elution, the protein was desalted using a 10-DG column (BioRad) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.8. The remaining proteins, ThiF (pET22), NifS (pET16), ThiO (pET22), ThiE (pQE32 and pREP4), TenI (pET28b), H122Q TenI (pET28b), and H122A TenI (pET28b), were overexpressed and purified in a similar manner. NifS, ThiO, and ThiE were stored in aliquots at  $-80$   $^{\circ}$ C in 20% glycerol. ThiSG, TenI, and ThiF were purified immediately before use.

**Reconstitution of the Thiazole Synthase-Catalyzed Reaction on an Analytical Scale (in the Presence and Absence of TenI).** All solutions were made with 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8. Final concentrations of the reactants are given in parentheses. Cysteine (0.35 mM), DTT (0.70 mM), ATP (0.60 mM), and MgCl<sub>2</sub> (3.5 mM) were incubated with purified ThiSG (1.25  $\mu$ M), ThiF (1.24  $\mu$ M), and 70  $\mu$ L of NifS (1.38  $\mu$ M) for 1.5 h. Total volume of this solution was 425  $\mu$ L. Glycine (6.50 mM), 1-deoxy-D-xylulose-5-phosphate (DXP) (0.33 mM), MgCl<sub>2</sub> (3.5 mM), and ThiO (6.8  $\mu$ M) were then added to this reaction mixture, and the final volume of the reconstitution mixture now was 610  $\mu$ L. TenI was added in the reconstitution reaction to a final concentration of 10  $\mu$ M to check for the acceleration of the rate of thiazole formation. In a control reaction set up in exactly the same way, the same volume of buffer was added into the reaction instead of TenI. This mixture was incubated for an additional 2 h. The reaction mixture was then analyzed for product formation using the thiochrome assay (see below). In this reconstitution, 16% of the DXP was converted to product. This is a 3-fold improvement over our previously reported reconstitution, and corresponds to about 12 turnovers by the thiazole synthase.

**Thiochrome Assay.**<sup>3,4</sup> The thiochrome assay involves conversion of the thiazole product of the reconstitution to thiamin phosphate 18 and further to thiochrome phosphate. The product of the thiazole reconstitution is reacted with HMP-PP 17 (0.5 mM) in the presence of thiamin phosphate synthase (ThiE) (1.00  $\mu$ M). The reaction is allowed to stand at room temperature for 2 h and then quenched with an equal volume of 10% TCA. Potassium acetate (50  $\mu$ L of 4 M) is added to 100  $\mu$ L of the quenched reaction followed by oxidative cyclization to thiochrome phosphate using 50  $\mu$ L of a saturated solution of K<sub>3</sub>Fe(CN)<sub>6</sub> in 7 M NaOH. The oxidation reaction is neutralized after 1 min with 6 M HCl and analyzed by reverse phase HPLC with fluorescence detection (excitation at 365 nm, emission at 450 nm). The following linear gradient, at a flow rate of 1 mL/min, was used. Solvent A is water, solvent B is 100 mM K<sub>2</sub>HPO<sub>4</sub>, pH 6.6, solvent C is methanol. 0 min: 100% B; 2 min: 10% A, 90% B; 10 min: 25% A, 15% B, 60% C; 12 min: 25% A, 15% B, 60%; 15 min: 100% B; 17 min: 100% B.

**ThiO Activity Assay.**<sup>16</sup> Twenty-five milliliters of assay solution containing 4 mM phenol, 100 mM 4-amino-antipyrine, and 2 units/mL horseradish peroxidase (HRP) was made. To 500  $\mu$ L of the assay solution was added 10 mM, 5 mM, 1 mM, 500  $\mu$ M, 250  $\mu$ M, or 100  $\mu$ M glycine, and the volume each time was diluted to 505  $\mu$ L. In each case the reaction was initiated by the addition of a final concentration of 6.6  $\mu$ M ThiO and 10  $\mu$ M TenI. A parallel set of reactions was similarly run in the absence of TenI. The rate of glycine oxidation was measured at the various glycine concentrations by monitoring the absorbance change at 500 nm for 600 s.

**ThiE Activity assay.**<sup>17</sup> In a final volume of 700  $\mu$ L of 50 mM Tris-HCl buffer, 2 mM MgCl<sub>2</sub>, pH 7.8, were mixed 437  $\mu$ M of Thz-P and 485  $\mu$ M HMP-PP with 10  $\mu$ M ThiE and 10  $\mu$ M TenI. An identical reaction lacking TenI was run under the same conditions. Aliquots of 100  $\mu$ L of each reaction mixture were quenched after 0, 0.5, 1, 2, 5, 10, and 20 min and oxidized to thiochrome phosphate. Reaction mixtures were analyzed by HPLC with fluorescence detection.

**ThiG Activity Assay.**<sup>3,4</sup> To evaluate the effect of TenI on ThiG, the thiazole reconstitution reaction was run using preformed ThiS-COSH in the presence and absence of TenI. Cysteine (0.35 mM), dithiothreitol (DTT) (0.70 mM), ATP (0.60 mM), and MgCl<sub>2</sub> (3.5 mM) were incubated with purified ThiSG (1.25  $\mu$ M), ThiF (1.24  $\mu$ M), and NifS (1.38  $\mu$ M) for 1.5 h. The total volume of this solution was 425  $\mu$ L. Desalting through a 10-DG column (BioRad) pre-equilibrated with 50 mM Tris-HCl, pH 7.8, yielded ThiS-COSH. Two sets of the thiazole reconstitution reactions were carried out as described above, one in the presence of TenI and the other in its absence. Aliquots of 100  $\mu$ L of these reaction mixtures were quenched at time points of 0, 1, 2, 5, and 10 min and oxidized to thiochrome phosphate which was detected by reverse phase HPLC with fluorescence detection.

**Activity Assay for TenI with cThz\*-P.** cThz\*-P was obtained as described in Supporting Information (Figures 4 and 5, Supporting Information). In a final assay volume of 300  $\mu$ L of 50 mM Tris buffer, pH 7.6, was added 1  $\mu$ M TenI to 100  $\mu$ M cThz\*-P. The mixture was incubated for 120 min, filtered using a 10 kDa MW cutoff filter, and analyzed by HPLC. Mutants were similarly analyzed except the reaction time was increased to 12 h.

**HPLC Conditions for Separation of cThz\*-P and cThz-P Using Analytical Strong Anion-Exchange Chromatography.** The following linear gradient, at a flow rate of 1 mL/min, on the Phenosphere Strong Anion-Exchange 80A (250 mm  $\times$  4.6 mm, 5  $\mu$ m ID) column was used: solvent A is water, solvent B is 100 mM triethylammonium acetate, pH 7.8; 0 min: 100% A; 1 min: 100% A; 4 min: 100% B; 7 min: 100% A; 10 min: 100% A.

**HPLC conditions for Separation of cThz\* and cThz Using Analytical Reverse Chromatography.** The following linear gradient, at a flow rate of 1 mL/min, on the Supelcosil LC-18-T column (150 mm  $\times$  4.6 mm, 3  $\mu$ m ID) was used: solvent A is water, solvent B is



100 mM KPi, pH 6.6, solvent C is methanol; 0 min: 100% B; 2 min: 100% B; 4 min: 10% A, 90% B; 9 min: 10% A, 25% B, 65% C; 14 min: 10% A, 25% B, 65% C; 16 min: 100% B; 20 min 100% B.

**Equilibration of cThz-P/cThz\*-P in the Presence of TenI.** To 540  $\mu$ M cThz-P (TenI/cThz-P ratio approximately 1:1) was added 540  $\mu$ M TenI in 50 mM potassium phosphate buffer (KPi) pH 7.6 in 50% D<sub>2</sub>O/H<sub>2</sub>O in a total volume of 650  $\mu$ L. The reaction mixture was analyzed by NMR after 12 h.

**Expression and Purification of *Bacillus subtilis* TenI for Crystallography.** The TenI overexpression strain was grown at 37 °C with vigorous agitation (200 rpm) in LB medium containing 30  $\mu$ g/mL kanamycin to an OD<sub>600</sub> of 0.7, at which point the cells were induced with 500  $\mu$ M IPTG and allowed to incubate overnight at 22 °C under conditions of mild mixing (180 rpm). The cells were harvested by centrifugation (6000g) for 15 min at 4 °C and stored at -80 °C for later use. All purification steps were carried out at 4 °C. The cell pellet was suspended in 50 mL of binding buffer (50 mM sodium phosphate, pH 7.0, and 300 mM NaCl) and lysed by sonication. The crude extract was centrifuged at 4 °C for 30 min at 50,000g, and the resulting supernatant was augmented with 5 mM imidazole and loaded onto a column containing 2 mL of TALON metal affinity resin equilibrated with 50 mL of binding buffer. The column was washed with 20 column volumes of binding buffer plus 5 mM imidazole, followed by 5 column volumes of binding buffer plus 10 mM imidazole. The His<sub>6</sub>-tagged TenI was eluted from the column with elution buffer (50 mM sodium phosphate, pH 7.0, 300 mM NaCl and 300 mM imidazole). The recombinant TenI was further purified on a Superdex 200 gel-filtration column and eluted in the storage buffer (25 mM Tris-HCl, pH 8.5, 150 mM NaCl, and 1 mM thiamin phosphate). The fractions containing pure TenI were combined and concentrated to 12 mg/mL using a 10 kDa cutoff concentrator (Vivaspin) and stored at -80 °C. Protein concentration was determined by the Bradford method with bovine serum albumin as the standard. The purity of TenI was determined by SDS-PAGE analysis and found to be greater than 99%.

**Crystallization of TenI with the cThz-P 16 Bound in the Active Site.** Crystals of ligand-free TenI were grown from 1.65 to 1.75 M ammonium sulfate, 100 mM bicine, pH 8.7–9.6, 2% PEG400 (w/v), and 8 mM L-cysteine using the hanging drop vapor diffusion method.<sup>12</sup> To obtain the product complex, the crystals were first dialyzed into 2.38 M sodium malonate (pH 7.0) (Hampton Research) to remove the sulfate ions by gradually increasing the sodium malonate concentration and decreasing the ammonium sulfate concentration in 30 steps with 5-min incubation for each step. Subsequently the crystals were soaked overnight in 2.38 M sodium malonate, 21.5 mM bicine (pH 9.0), 0.01% PEG400 (w/v), 0.5 mM L-cysteine, 4% glycerol, and 11.5 mM cThz-P, followed by flash freezing in liquid nitrogen.

**X-ray Data Collection and Processing.** The X-ray intensity data from the TenI–cThz-P complex were measured at the A1 beamline of the Cornell High Energy Synchrotron Source (CHESS) using a Quantum 210 CCD detector (Area Detector Systems Corp.). Data were collected over 180° using a 10-s exposure time and 1° oscillation per frame with a crystal-to-detector distance of 200 mm. The data were integrated and scaled using HKL2000.<sup>23</sup>

**Structure Determination and Refinement.** The structure of TenI–cThz-P complex was determined by Fourier synthesis using the previously reported TenI structure (PDB: 1YAD) as the starting model. The first round of rigid-body refinement with the starting model by REFMACS<sup>24</sup> reduced the *R*-factor to 0.286 (*R*<sub>free</sub> 0.294). cThz-P was modeled based on clear electron density. The model was refined through iterative cycles of restrained refinement by REFMACS and PHENIX,<sup>25</sup> and manual rebuilding in COOT.<sup>26</sup> Refinement statistics are shown in Table 1, Supporting Information.

**Modeling of cThz\*-P in the Active Site.** The initial coordinates of proposed intermediate cThz\*-P were<sup>26</sup> generated using program

SKETCHER of CCP4 suite.<sup>27</sup> Using the crystal structure of TenI complexed with Thz-P as the template, the intermediate cThz\*-P was docked into the active site, and the key interacting residues were adjusted manually in COOT.<sup>26</sup> Stereo figures were produced with PyMOL.<sup>28</sup>

## ■ ASSOCIATED CONTENT

**Supporting Information.** Figures for the gene neighborhood for TenI and TenA, comparison of the primary sequence of TenI with ThiE, time course for effect of TenI on thiazole reconstitution, procedure for preparing and assaying cThz\*-P, NMR analysis of cThz\*-P decomposition, mass spectrum of cThz-P and cThz\*-P, and HPLC analysis of TenI mutants with cThz\*-P, and cThz-P/TenI crystal structure data and refinement statistics. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## Accession Codes

<sup>†</sup>The coordinates of the TenI–cThz-P complex have been deposited in the Protein Data Bank under accession number 3QH2.

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## ■ ABBREVIATIONS:

Thz-P, 2-(4-methylthiazol-5-yl)ethyl phosphate; cThz-P, 2-(2-carboxy-4-methylthiazol-5-yl)ethyl phosphate; cThz\*-P, (*R,Z*)-2-(2-carboxy-4-methylthiazol-5(2*H*)-ylidene)ethyl phosphate; cThz, 5-(2-hydroxyethyl)-4-methylthiazole-2-carboxylic acid; cThz\*, (*R,Z*)-5-(2-hydroxyethylidene)-4-methyl-2,5-dihydrothiazole-2-carboxylic acid; cThz-ADP, adenylated 2-(2-carboxy-4-methylthiazol-5-yl)ethyl phosphate; cThz\*-ADP, adenylated (*R,Z*)-2-(2-carboxy-4-methylthiazol-5(2*H*)-ylidene)ethyl phosphate; HRP, horse radish peroxidase; DXP, 1-deoxy-D-xylulose-5-phosphate; HMP-P, 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate; HMP-PP, 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; KPi, potassium phosphate buffer; NTA, nitrilotriacetic acid; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; ThiS, ThiF, NifS, ThiG, ThiO, thiazole biosynthetic proteins; TenA, thiaminase II; ThiE, thiamin phosphate synthase; *tenI*, gene of unknown function; TenI, corresponding protein of unknown function

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