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The Biosynthesis of the Thiazole Phosphate Moiety of Thiamin: The Sulfur Transfer Mediated by the Sulfur Carrier Protein ThiS

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Summary

Thiamin-pyrophosphate is an essential cofactor in all living systems. The biosynthesis of both the thiazole and the pyrimidine moieties of this cofactor involves new biosynthetic chemistry. Thiazole-phosphate synthase (ThiG) catalyses the formation of the thiazole moiety of thiamin-pyrophosphate from 1-deoxy-D-xylulose-5-phosphate (DXP), dehydroglycine and the sulfur carrier protein (ThiS), modified on its carboxy terminus as a thiocarboxylate (ThiS-thiocarboxylate). Thiazole biosynthesis is initiated by the formation of a ThiG/DXP imine, which then tautomerizes to an amino-ketone. In this paper we study the sulfur transfer from ThiS-thiocarboxylate to this amino-ketone and trap a new thioenolate intermediate. Surprisingly, thiazole formation results in the replacement of the ThiS-thiocarboxylate sulfur with an oxygen from DXP and not from the buffer, as shown by electrospray ionization Fourier transform mass spectrometry (ESI-FTMS) using ^{18}O labeling of the ^{13}C-, ^{15}N-depleted protein. These observations further clarify the mechanism of the complex thiazole biosynthesis in bacteria.

Introduction

Thiamin pyrophosphate (**9**, Figure 1) is an essential cofactor in all living systems and consists of a pyrimidine covalently linked to a thiazole. This cofactor is utilized for reactions catalyzed in amino acid metabolism, the pentose phosphate pathway, and the citric acid cycle. [1, 2] Furthermore, thiamin-triphosphate has been implicated in brain disorders [2]. In thiamin deficient humans, these processes do not function properly and result in disease states known as Beri-Beri or Werninke-Korsakoff syndrome, both of which can be fatal [2]. Recently (2003), fatal Beri-Beri was diagnosed in infants from Jewish communities in Israel as a result of the consumption of thiamin-deficient baby-food products [4]. Considering the important cellular roles thiamin plays, it is surprising that we are now just beginning to understand its biosynthesis in bacteria, while its biosynthesis in eukaryotes is still at an early stage [3, 5–7]. In this paper, we characterize the mechanism of formation of the thiazole-phosphate moiety (**8**) of thiamin in vitro starting with the sulfur transfer reaction from ThiS-thiocarboxylate to the amino ketone **5**, during which a hydroxyl group from 1-deoxy-D-xylulose-5-phosphate (**1**) (DXP) is transferred to the C-terminal end of the sulfur carrier protein, ThiS-carboxylate. This was unambiguously shown using high resolution electrospray ionization Fourier mass spectrometry (ESI-FTMS) of the undigested ^{13}C- and ^{15}N-depleted protein formed from ^{18}O-labeled DXP. In addition, we have trapped and identified a new DXP-derived thioenolate intermediate covalently linked to ThiG. We propose a mechanism for the complex biosynthesis of the thiazole-phosphate moiety based on these findings.

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Results and Discussion

The thiazole moiety **8** (Figure 1) is biosynthesized in *Bacillus subtilis* and most other bacteria from 1-deoxy-D-xylulose-5-phosphate (**1**, DXP), glycine, and cysteine in a complex oxidative condensation reaction [5]. This reaction requires five different proteins, ThiO, ThiG, ThiS, ThiF, and a cysteine desulfurase. Glycine oxidase (ThiO) catalyzes the oxidation of glycine to the corresponding imine **7**, sulfur carrier protein adenylyl transferase (ThiF) catalyzes the adenylation of the carboxy terminus of the sulfur carrier protein (ThiS-carboxylate), and cysteine desulfurase catalyzes the transfer of sulfur from cysteine to the ThiS-acyl adenylate to give ThiS-thiocarboxylate (**6**) [5, 8, 9, 19]. ThiG is the thiazole synthase and catalyzes the formation of the thiazole from dehydroglycine **7**, DXP **1**, and ThiS-thiocarboxylate **6**. The early steps in thiazole formation have been elucidated [10]: Imine formation between lysine 96 on ThiG and DXP followed by tautomerization gives amino-ketone **5**, which is then proposed to react with ThiS-thiocarboxylate **6** and dehydroglycine **7** to give thiazole phosphate **8**.

During the formation of thiazole-phosphate (**8**), intermediate **10** formed by the addition of **6** to the C3 of **5**, could undergo hydrolysis, releasing ThiS-carboxylate, followed by loss of water to give thioketone **12** (mechanism A, Figure 2). Enolization of **12** would give **13**, which could react with the dehydroglycine **7** to give the thiazole **8** or eliminate water to give **14**, which could then react with the dehydroglycine. Alternatively, sulfur to oxygen acyl shift in **10** would give **15**, loss of water would give thioketone **16**, which could then enolize to give **17** (mechanism B, Figure 2). This could react with the dehydroglycine **7** to give the thiazole **8** or eliminate ThiS-carboxylate to give **14**, which could in turn react with the dehydroglycine. The experiments reported here allow us to differentiate between mechanisms A and B.

Determination of the Source of the Carboxy-Terminal Oxygen of ThiS-Carboxylate Derived from 6 by ESI-FTMS

One can differentiate between mechanisms A and B in Figure 2 by determining the origin of the oxygen on ThiS-carboxylate that replaced the sulfur of ThiS- thiocarboxylate (**6**). For mechanism A, this oxygen will be derived from the buffer, whereas for mechanism B, it will be derived from DXP.

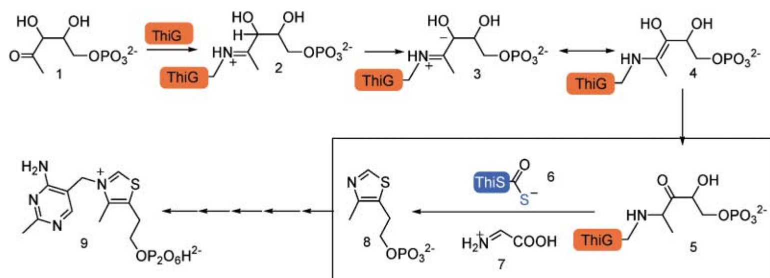

Figure 1. The Biosynthesis of the Thiazole-Phosphate Moiety (**8**) of Thiamin Pyrophosphate (**9**) in *B. subtilis*

To determine the origin of this oxygen, ThiS-thiocarboxylate (**6**) and DXP (**1**) were incubated with ThiG in ^{18}O buffer and the resulting ThiS-carboxylate was analyzed by ESI-FTMS to give the spectrum shown in Figure 3B. The observed molecular ion was 10146.4 Da, identical to the calculated mass of ThiS-carboxylate (Figure 3C), suggesting that ^{18}O from buffer was not incorporated into ThiS-carboxylate. When a similar reaction was run in ^{16}O buffer using partially labeled [2,3,4-^{18}O]-DXP (prepared using dihydroxyacetone-phosphate, pyruvate, triose-phosphate isomerase, and DXP-synthase in 70% ^{18}O-buffer [10, 11]), the corresponding molecular ion had a mass between 10146.4 and 10148.4 Da (Figure 3A), suggesting that the new carboxy-terminal oxygen of ThiS-carboxylate was derived from DXP rather than from the buffer. We have obtained further support for this conclusion using ^{13}C-, ^{15}N-depleted ThiS-thiocarboxylate as described below.

Confirmation of the Source of the Carboxy-Terminal Oxygen Derived from 6 by ESI-FTMS Using ^{13}C-, ^{15}N-Depleted ThiS-Thiocarboxylate

To simplify the mass spectrum of ThiS-carboxylate shown in Figure 3A, we were able to reduce the ThiS-carboxylate isotopic cluster to a single major species using ^{13}C-, ^{15}N-depleted ThiS-carboxylate [12]. To accomplish this, ^{13}C-, ^{15}N-depleted ThiS-carboxylate was overexpressed and purified from minimal medium containing ^{13}C-depleted glucose and ^{15}N-depleted ammonium sulfate. The mass spectrum of this protein is shown in Figure 4B and is compared to the mass spectrum of native ThiS-carboxylate in Figure 4A. The monoisotopic ion, with an observed mass of 10140.3 Da, is the major species. ^{13}C-, ^{15}N-depleted ThiS-carboxylate was enzymatically converted to ^{13}C-, ^{15}N-depleted ThiS-thiocarboxylate. The mass spectrum of this protein is shown in Figure 4C and shows the expected +16 Da mass increase. When ^{13}C-, ^{15}N-depleted ThiS-thiocarboxylate and [3,4-^{18}O]-DXP (prepared using dihydroxyacetone-phosphate, pyruvate, triose-phosphate isomerase, and DXP-synthase in 80% ^{18}O-buffer) were incubated with ThiG in ^{16}O-buffer, the resulting ^{13}C-, ^{15}N-depleted ThiS-carboxylate had a measured mass of 10142.3 Da, 2 Da larger than ^{13}C-, ^{15}N-depleted ThiS-carboxylate (Figure 4D), the calculated mass for which is 10142.1 Da. The amount of ^{18}O-label incorporated into ThiS-carboxylate in this experiment was estimated to be 78% (data not shown). In contrast, an identical reaction in which [3,4-^{18}O]-DXP was replaced with DXP, without ^{18}O-labeled oxygens, gave ^{13}C-, ^{15}N-depleted ThiS-carboxylate with the mass spectrum shown in Figure 4E, similar to the starting ^{13}C-, ^{15}N-depleted ThiS-carboxylate shown in Figure 4B. SWIFT isolation and IR-multiphoton dissociation of the reformed ThiS-carboxylate ion observed in Figures 3A and 4D resulted in fragmentation at 22 different sites. None of the ***b*** fragments (amino terminal) containing residues 84, 85, and 86 of the 87 amino-acid-reformed ThiS-carboxylate carried the ^{18}O label (+2 Da modification) while all the ***y*** fragments (carboxy terminal) did (Figures 4F and 4G). While this experiment does not exclude the possibility that the oxygen replacing the sulfur of ThiS-thiocarboxylate is derived from the C3 rather than the C4 oxygen of DXP, the experiment unambiguously demonstrates that the oxygen of the reformed ThiS-carboxylate is derived from DXP and not from the buffer. Therefore mechanism A in Figure 2 can be excluded from further consideration.

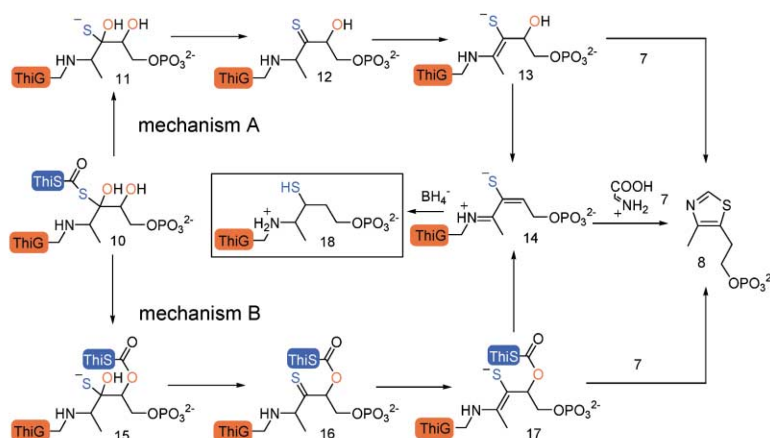

Figure 2. Mechanistic Analysis of the Middle Steps in the Formation of Thiazole Phosphate

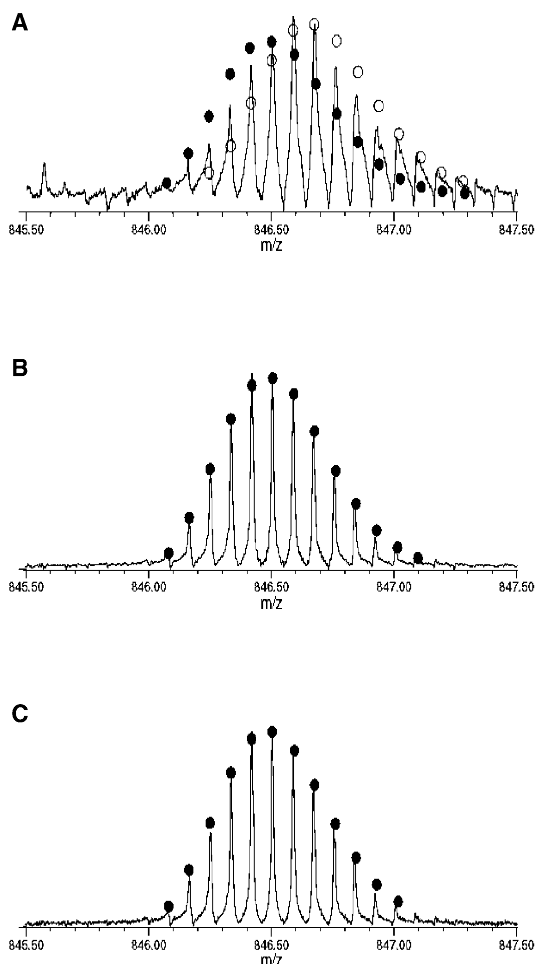

Figure 3. Identification of the Source of the New Carboxy-Terminal Oxygen on ThiS-Carboxylate by ESI-FTMS Analysis

(A) Mass spectrum of ThiS-carboxylate derived from the reaction of ThiS-thiocarboxylate with amino-ketone **5** formed using [2,3,4-^{18}O]-DXP in ^{16}O buffer. Closed circles: predicted spectrum of ThiS-carboxylate; open circles: predicted spectrum of ThiS-carboxylate with a single ^{18}O incorporated.

(B) Mass spectrum of ThiS-carboxylate derived from the reaction of ThiS-thiocarboxylate with amino-ketone **5** formed using DXP in ^{18}O buffer.

(C) Mass spectrum of a reference sample of ThiS-carboxylate. The charge state for the molecular ion shown is +12.

Detection by ESI-FTMS of the DXP-Derived Thioenolate 14 Covalently Attached to ThiG

The observation that ThiG catalyzes the formation of ThiS-carboxylate (**21**, Figure 5A) from ThiS-thiocarboxylate (**6**) in the absence of dehydroglycine (**7**) suggests that **17** undergoes an elimination to give **14** before reaction with the dehydroglycine occurs (Figure 2). Putative intermediate **14** was expected to hydrolyze to **20** in the absence of the dehydroglycine **7** (Figure 5A). To probe for the hydrolysis of **14**, a reaction mixture generated by treating ThiG and ThiS-thiocarboxylate with DXP was initially monitored by ESI-FTMS for the conversion of ThiS-thiocarboxylate (**6**) to ThiS-carboxylate **21** (Figures 5B and 5C). After the sulfur transfer reaction had gone to completion, the reaction mixture was reduced with $NaBH_4$ and the resulting ThiG was analyzed by ESI-FTMS for covalent modification (Figure 5D). The mass of the resulting ThiG molecular ion (27004 Da) was 200 Da larger than that of native ThiG (26804 Da). This is not consistent with the trapping of **20** because the mass of reduced **20** is ThiG + 184 Da. The observed mass increase of +200 Da is consistent with reduced **5** (Figure 6A) and with reduced **14.** To explain the absence of **20**, it is possible that **14** exists primarily in the thioenol tautomeric form at the active site, and is therefore not hydrolyzed because a thioenol cannot be hydrolyzed unless it first forms the corresponding thioketone. In support for this hypothesis, it has been observed that in contrast to ketones, thioenolates are more stable than thioketones [13].

To differentiate between reduced **14** and reduced **5** (Figure 5A), it was necessary to determine if the +200 Da trapped intermediate contained sulfur. Two experimental strategies addressed this issue. Our first approach involved repeating the trapping reaction using [^{35}S]-ThiS-thiocarboxylate (**6**). If the trapped intermediate contains the sulfur from ThiS-thiocarboxylate, we would expect to see radioactivity transfer from [^{35}S]-ThiS-thiocarboxylate to ThiG, which could be detected by SDS-PAGE/autoradiography. The gel shown in Figure 6E clearly demonstrated the incorporation of [^{35}S] into ThiG. When the borohydride reduction step was omitted, the radiolabel was lost from ThiG because the imine of thioenolate **14** is hydrolyzed from ThiG under the denaturing SDS-PAGE conditions (data not shown). When less than 1 equivalent of DXP is used with respect to [^{35}S]-ThiS-thiocarboxylate and ThiG, ~95% of the radioactivity lost from ThiS is transferred to ThiG.

Our second approach to detecting the presence of an additional thiol in the +200 Da trapped intermediate involved thiol alkylation with iodoacetamide followed by MS analysis. The results of this experiment are shown in Figures 5F–5I. Native ThiG and borohydride-reduced ThiG/DXP imine (reduced **5**) are both monoalkylated by iodoacetamide via a solvent-exposed cysteine, increasing the observed mass by 57 Da (Figures 5G and 5I). In contrast, ThiG covalently linked to the trapped intermediate is dialkylated, increasing its observed mass by 114 Da (Figures 5F and 5H), demonstrating the presence of an extra thiol on the trapped intermediate. The ^{35}S transfer and the iodoacetamide alkylation experiments are both consistent with the assignment of the trapped intermediate as reduced **14** and not reduced **5**.

Catalytic Competence of the Sulfur-Containing Thioenolate 14

Since the thioenolate (**14**)-forming reaction was run for several hours before reduction with borohydride, it is possible that **14** might be a decomposition product of **17** (Figure 2) and not a true intermediate. It was therefore necessary to test the catalytic competence of **14** in the thiazole-forming reaction. This was accomplished by monitoring the sulfur transfer from **14** to form the thiazole **8** in the presence of dehydroglycine (**7**). Thus **14** was prepared, as described above, and treated with dehydroglycine (**7**, prepared in situ by ThiO-catalyzed oxida-

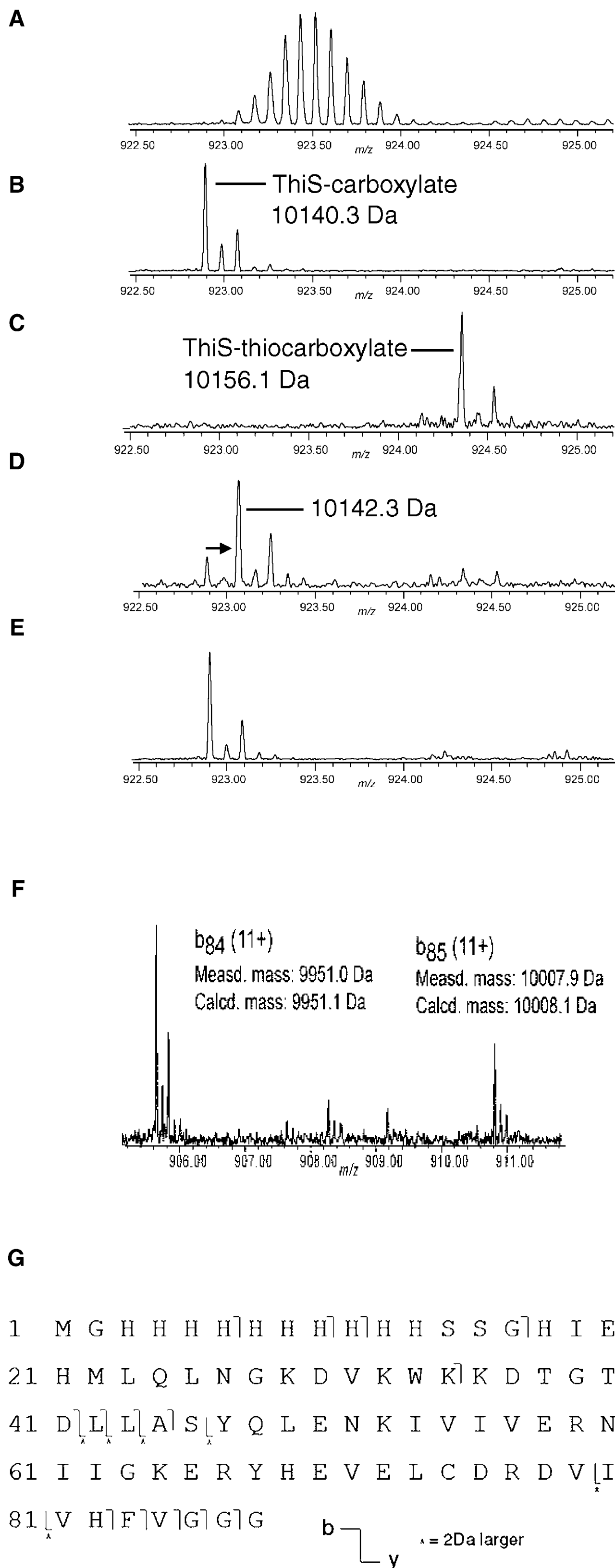

Figure 4. Identification by ESI-FTMS, of the Source of the Carboxy-Terminal Oxygen on the Reformed ThiS-Carboxylate Generated by Reacting ^{13}C-, ^{15}N-depleted ThiS-Thiocarboxylate with Amino-Ketone **5**

(A) Mass spectrum of native ThiS-carboxylate.

(B) Mass spectrum of ^{13}C-, ^{15}N-depleted ThiS-carboxylate.

(C) Mass spectrum of ^{13}C-, ^{15}N-depleted ThiS-thiocarboxylate.

(D) Mass spectrum of ^{13}C-, ^{15}N-depleted ThiS-carboxylate derived from the reaction of ^{13}C-, ^{15}N-depleted ThiS-thiocarboxylate with amino-ketone **5** formed using [2,3,4-^{18}O]-DXP in ^{16}O buffer.

(E) Mass spectrum of ^{13}C-, ^{15}N-depleted ThiS-carboxylate derived from the reaction of ^{13}C-, ^{15}N-depleted ThiS-thiocarboxylate with amino-ketone **5** formed using DXP in ^{16}O buffer.

(F) The b_{84} and b_{85} fragments of the SWIFT isolated molecular ion of ^{13}C-, ^{15}N-depleted [^{18}O]-ThiS- carboxylate shown in Figure 4D do not contain the [^{18}O] label.

(G) A summary of all the fragments observed for reformed [^{18}O]-ThiS-carboxylate. The charge state for all molecular ions is +11. The arrow in (D) indicates the observed 2 Da shift in the mass of the reformed ThiS-thiocarboxylate.

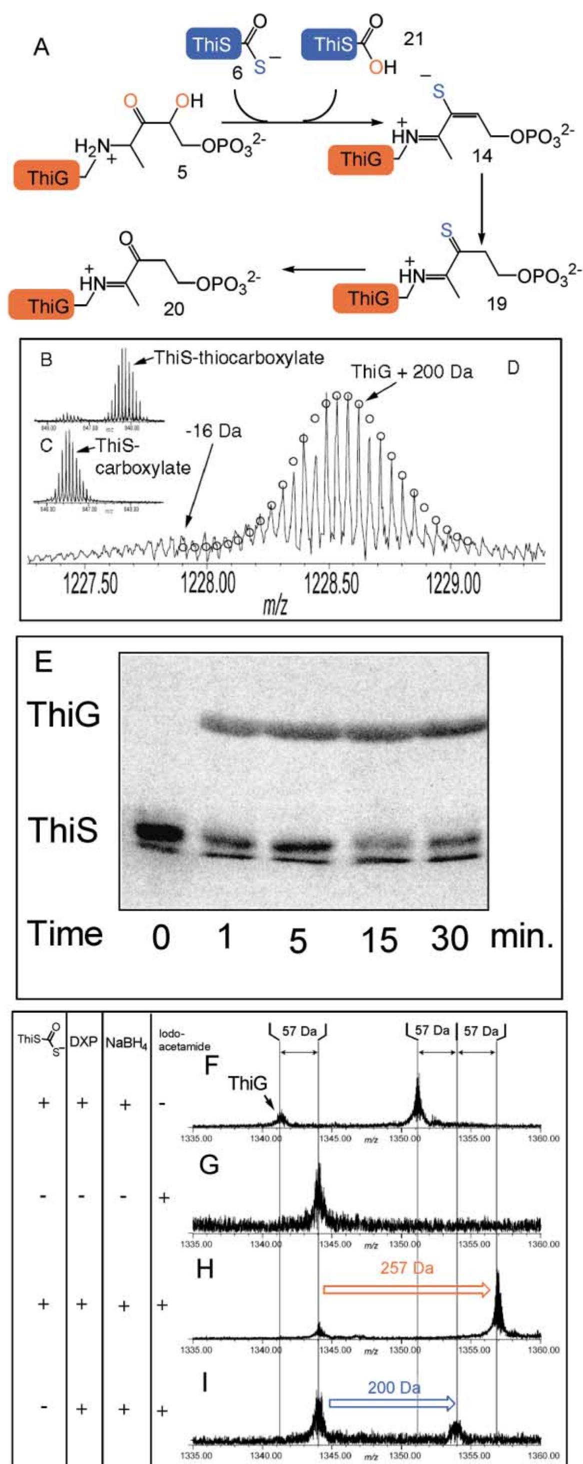

Figure 5. Sulfur Transfer from ThiS-Thiocarboxylate to the ThiG-Amino-Ketone (**5**)

(A) Mechanistic scheme for the formation of the putative hydrolysis product (**20**).

(B) MS of ThiS-thiocarboxylate (**6**).

(C) MS of ThiS-carboxylate (**21**) formed by incubating ThiS-thiocarboxylate (**6**) with ~**1** equivalent of [1-^{13}C]-DXP and ThiG.

(D) The molecular ion of the borohydride trapped intermediate (mass = 27,004 Da), which is consistent with reduced **5** or **14**. The open circles give the best theoretical fit to the data. The charge state for this molecular ion is +22.

(E) SDS-PAGE analysis of the sulfur transfer from [^{35}S]-ThiS-thiocarboxylate (**6**) to the ThiG-amino-ketone **5** followed by borohydride reduction. t = 0 min is before the addition of DXP. The [^{35}S]-ThiS-thiocarboxylate contains a minor proteolysis product.

(F) The molecular ions of ThiG from a borohydride-treated reaction mixture containing ThiS-thiocarboxylate, ThiG, and DXP.

(G) The molecular ions of ThiG when treated with iodoacetamide.

(H) Iodoacetamide-treated reaction mixture F.

(I) The molecular ions of ThiG + DXP following borohydride reduction and alkylation by iodoacetamide. The proposed structure for the ThiG + 200 Da adduct observed in (F) is the reduced structure **18** in Figure 2.

tion of glycine, Figure 6A). This reaction was quenched by borohydride reduction and the residual [^{35}S]-thioenolate-ThiG (**14**) was measured by SDS-PAGE/autoradiography. In Figure 6B, lanes 3–7 show the time-dependent loss of [^{35}S] from [^{35}S]-thioenolate-ThiG following the addition of ThiO and glycine. The reaction does not go to completion for reasons that we do not yet understand. When glycine is omitted from the reaction mixture, no loss of [^{35}S] from labeled ThiG is observed (Figure 6B, lane 8). To demonstrate that the [^{35}S] label lost from ThiG is converted to thiazole phosphate **8**, a similar reaction was run in the presence of HMP, ThiD, and ThiE followed by ferricyanide oxidation to convert any thiazole phosphate formed to the fluorescent thiochrome phosphate (Figure 6D) [5]. HPLC analysis of this reaction mixture, with product detection using in-line scintillation counting, demonstrated the formation of [^{35}S]-thiochrome phosphate from [^{35}S]-ThiS-thiocarboxylate (Figure 6C). The radiochemical yield of this complex multistep biosynthesis was 16%. This provides evidence that thioenolate **14** is an intermediate en route to thiazole-phosphate.

Based on these experiments, we can now expand our mechanistic proposal for thiazole biosynthesis to include the middle steps in the pathway (Figure 7). Thiazole biosynthesis is initiated by the formation of the ThiG/DXP imine **2**, which then tautomerizes to the amino-ketone **5**. Addition of ThiS-thiocarboxylate (**6**) to the C3 carbonyl group of **5** gives **10**. An S to O acyl shift followed by loss of water gives **16**. This is an interesting intermediate in which modified DXP covalently links both the thiazole synthase (ThiG) and the sulfur carrier protein (ThiS). Such complexes are very unusual in enzymology. While the labeling experiment does not differentiate between ThiS transfer to the C3 or the C4 oxygen of **10**, we feel that transfer to the C4 oxygen is more likely, because transfer to the C3 alcohol involves a strained four-membered ring transition state. Furthermore, we have not observed any reduced **12** (Figure 2), which would be 16 Da larger than reduced **2** or **14** by MS. If acyl transfer to the C3 oxygen occurred, we would expect to detect this intermediate. In contrast, acyl transfer to the C4 alcohol can proceed via a five-membered ring transition state and is likely to be facile. In addition, the formation of an ester with the C4 alcohol would facilitate the elimination of water from this position. Enolization followed by elimination of ThiS-carboxylate (**21**) gives **14**, which then reacts with the dehydroglycine to form the thiazole-phosphate **8**.

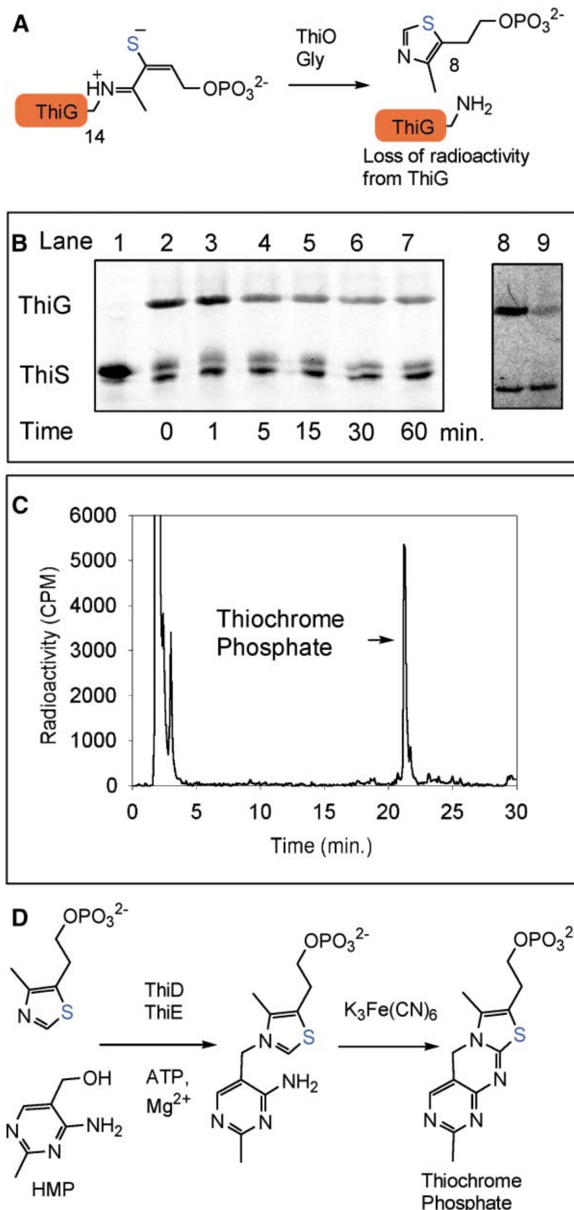

Figure 6. Conversion of Thioenolate **14** to Thiazole-Phosphate by the Addition of ThiO and Glycine

(A) The sulfur transfer reaction from the thioenolate **14** to form the thiazole phosphate **8**.

(B) Analysis of the dehydroglycine-dependent removal of ^{35}S from the [^{35}S]-thioenolate **14** by SDS-PAGE/autoradiography. Lane 1 shows the [^{35}S]-ThiS-thiocarboxylate/ThiG reaction mixture before the addition of DXP. Lane 2 shows the [^{35}S]-ThiS-thiocarboxylate/ThiG reaction mixture 30 min after the addition of DXP (**1**). Lanes 3–7 show the time course for the loss of sulfur from [^{35}S]-thioenolate-ThiG (lane 2) following the addition of ThiO and glycine. Lane 8 is the complete reaction mixture except glycine has been omitted (incubated for 120 min.). Lane 9 is the complete reaction mixture (incubated for 120 min).

(C) HPLC analysis of the thiochrome reaction mixture using in-line scintillation counting for the detection of [^{35}S]-thiochrome phosphate.

(D) An outline of the assay protocol for the conversion of [^{35}S]-thiazole phosphate to [^{35}S]-thiochrome phosphate.

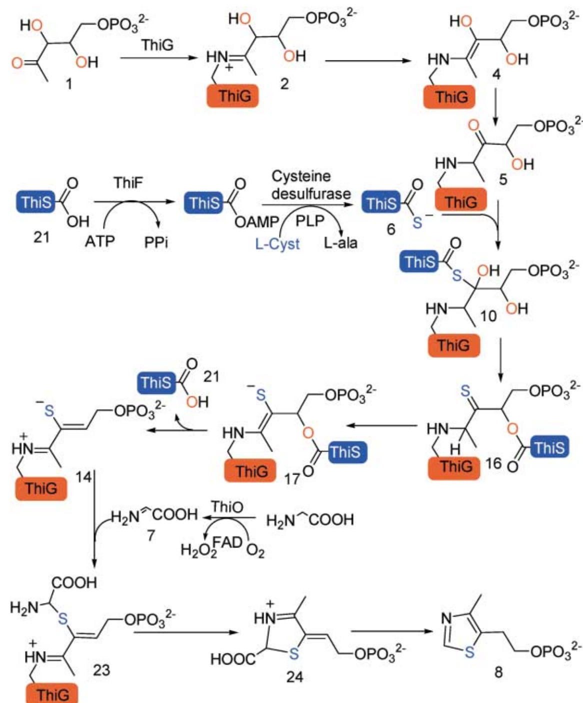

Figure 7. The Current Mechanistic Proposal for the Formation of Thiazole-Phosphate **8** in *B. subtilis*

Significance

Thiamin pyrophosphate is an essential cofactor in all living systems. The biosynthesis of the thiazole moiety of this cofactor is different from any of the numerous chemical syntheses of the thiazole heterocycle. It is also different from other known thiazole biosyntheses which typically proceed by the addition of a cysteinyl thiol to an adjacent amide. In contrast, the biosynthesis of the thiamin thiazole is biosynthesized from a protein thiocarboxylate (ThiS-COSH), 1-deoxy-D-xylulose-5-phosphate (DXP) and glycine in a reaction catalyzed by four enzymes (ThiF, ThiG, ThiO, and NifS). Here we demonstrate, using ESI-FTMS, that the sulfur of ThiS-COSH is replaced by an oxygen from ^{18}O-labeled DXP during thiazole formation and describe the identification of a new, sulfur-containing reaction intermediate on this complex biosynthetic pathway.

Experimental Procedures

General Methods

B. subtilis ThiS, ThiG, ThiF, NifZ, YrvO, and DXP synthase were overexpressed and purified as previously described [5]. The purity of each protein was assessed using 12% or 16% SDS-PAGE. Protein concentration was determined using the Bradford assay with BSA as the standard. The concentration of ThiG in samples where ThiS-carboxylate and ThiG were copurified was estimated with the assumption that ThiS-carboxylate and ThiG copurified as a 1:1 complex.

Materials

Dihydroxyacetone phosphate (DHAP, lithium salt), Pyruvate (sodium salt), fructose bis-phosphate and, ^{14}N ammonium sulfate (40% solu-

tion, 99.99%) were purchased from Sigma-Aldrich and U-^{12}C-glucose (99.9%) and ^{18}O water (98%) were purchased from Cambridge Isotope Laboratories. Bio-Spin 6 centrifugal gel filtration columns were from BIO-RAD and 3 kDa microcon membrane filters were form Millipore. TLC plates (Kieselgel 60 F_{254}) were from Merck. PD-10 size exclusion columns were from Amersham Biosciences. We used synthetic [1-^{13}C]-DXP for most experiments because this batch of DXP had the highest purity of all the DXP available in the lab [10].

Formation of ThiS-Thiocarboxylate

ThiS-thiocarboxylate was enzymatically prepared as previously described [5]. A typical procedure involved the copurification of ThiS-carboxylate, ThiG, and ThiF (purified from 1 liter of overexpressed ThiS-carboxylate/ThiG and 0.5 liter of overexpressed ThiF) followed by buffer exchange through a PD-10 column equilibrated in 20 mM Tris (pH 7.7). The buffer-exchanged proteins (1 ml of 5–10 mg/ml) were then incubated with 4 mM ATP, 8 mM $MgCl_2$, 2 mM Cysteine, 4 mM DTT and the cysteine desulfurases NifZ (120 μg) or YrvO (200 μg). The formation of ThiS-thiocarboxylate was complete within 1 to 2.5 hr as judged by ESI-FTMS. The proteins were then buffer exchanged on a PD-10 column (Amersham) into 20 mM Tris (pH 7.7).

Formation of [2,3,4-^{18}O]-DXP and [3,4-^{18}O]-DXP

The reaction mixture (200 μl) containing 10 mM DHAP, 50 mM Tris, 4 mM $MgCl_2$, and 2 mM DTT (pH 7.8) was lyophilized and redissolved in 70–80 μl ^{18}O water containing 200 units of triose-phosphate-isomerase [10, 11]. This was incubated at room temperature for 30 min to 12 hr. Pyruvate (20 mM), thiamine-pyrophosphate (10 μM), and DXP synthase (20–30 μL, 2–3 mg/ml) were then added. The reaction mixture was incubated at room temperature for 6–24 hr and the proteins were removed by ultrafiltration (3 kDa cutoff). The resulting mixture containing [2,3,4-^{18}O]-DXP was divided into 10 μl aliquots and frozen at −80°C until use. (ESI-MS, M^-) 213, 215, 217, and 219 Da, TLC analysis (6:1:3 N-propanol: ethyl actetate: water) Rf 0.6. DXP was visualized by dipping the plate into a ceric ammonia molybdenate solution and heating the plate to 300°C for 30–60 s until a blue spot appeared. To exchange the ^{18}O-labeled carbonyl oxygen, a sample was lyophilized and redissolved in 10 μl of $H_2{}^{16}O$. After 12 hr, the carbonyl oxygen was fully exchanged and the resulting solution was stored at −80°C. (ESI-MS, M^-) 213, 215, 217.

Conversion of ThiS-Thiocarboxylate to ThiS-Carboxylate in the Presence of [2, 3, 4-^{18}O]-DXP

To 500 μl of gel-filtered ThiS-thiocarboxylate/ThiG (typical concentration 5–10 mg/ml), prepared as described above, was added to 5 μl of [2,3,4-^{18}O]-DXP. The conversion of ThiS-thiocarboxylate to ThiS-carboxylate was monitored by ESI-FTMS.

Determination if the Oxygen of Reformed ThiS-Carboxylate Is Derived from the Buffer

Method A: ThiS-thiocarboxylate was preformed as described above except that it was buffer exchanged using a gel filtration column (Biospin 6) equilibrated in 80% ^{18}O-buffer (50 mM Tris-HCl and 2 mM DTT [pH 7.8]). After the addition of 200 μM unlabeled DXP, the conversion of ThiS-thiocarboxylate to ThiS-carboxylate was monitored by ESI-FTMS.

Method B: A solution of preformed ThiS-thiocarboxylate, ThiG, ThiF, and YrvO (in 50 mM Tris [pH 7.8]) prepared as described above was concentrated using a microcon concentrator (3 kDa cutoff). Forty microliters of this solution, with a total protein concentration of 32 mg/ml, was diluted into 160 μl $H_2{}^{18}O$. After the addition of 200 μM unlabeled DXP, the conversion of ThiS-thiocarboxylate to ThiS-carboxylate was monitored by ESI-FTMS.

Overexpression and Purification of ^{13}C-, ^{15}N-Depleted ThiS-Carboxylate

An overnight culture of a BL21(DE3) overexpression strain containing a plasmid encoding ThiS(His tagged) and ThiG, in LB medium with 50 μg/ml of ampicillin (5 ml) was spun down in a clinical centrifuge and the supernatant was discarded [5]. The cell pellet was washed three times with wash buffer (5 ml, 3 g KH_2PO_4, 6 g Na_2HPO_4, and 0.5 g NaCl per liter [pH 7.4]), resuspended in wash buffer (2 ml), and added to 300 ml of modified M9 minimal media (pH 7.4) containing 1 g KH_2PO_4, 2 g Na_2HPO_4, 0.2 g NaCl, 0.8 ml of 40% $(^{14}NH_4)_2SO_4$, 0.55 g of ^{13}C-depleted glucose, 0.2 mg of $FeCl_3$, 20 μg thiamin, 400 μl of 1 M $MgSO_4$, 20 μl of 1 M $CaCl_2$, 50 μg/ml of ampicillin, and 300 μl of a trace element solution containing per liter 0.55 g $CaCl_2$, 0.17 g $ZnCl_2$, 0.043 g of $CuCl_2.H_2O$, 0.06 g of $CoCl_2.6H_2O$, and 0.06 g of $Na_2MoO_4.2H_2O$ (pH 7.4). The cells were induced at OD_{595} = 0.76 (after approximately 4 to 5 hr) by the addition of 50 mg of IPTG and grown with agitation at 37°C for an additional 7 hr. The culture was then combined with a 100 ml culture of the ThiF overexpression strain grown in LB/amp medium and the cells were harvested by centrifugation. The cell pellet containing overexpressed ^{13}C-, and ^{15}N-depleted ThiS-carboxylate/ThiG and normal ThiF was frozen at −80°C until use.

Preparation of ^{13}C-, ^{15}N-Depleted ThiS-Thiocarboxylate

^{13}C-, ^{15}N-depleted ThiS-carboxylate, ThiG, and unlabeled ThiF, prepared as described above (200 μl), were purified and buffer exchanged into 20 mM Tris (pH 7.8). One hundred microliters of this sample was converted into ThiS-thiocarboxylate by the addition of 4 mM ATP, 8 mM $MgCl_2$, 2 mM cysteine, 4 mM DTT, and NifZ (6 μg). After 1.5 hr (>97% completion judged by ESI-FTMS), the reaction mixture was buffer exchanged into 20 mM Tris, 0.02% sodium azide (pH 7.4) using a Biospin column and used immediately.

Incorporation of ^{18}O from [3,4-^{18}O]-DXP into Nascent ThiS-Carboxylate

[3,4-^{18}O]-DXP (0.5 μl, prepared with DXP synthase in 80% [^{18}O]-H_2O and 10 mM DHAP and 20 mM pyruvate described above) was added to 100 μl of the ^{13}C-, ^{15}N-depleted ThiS-thiocarboxylate sample prepared as described above, and the conversion of ThiS-thiocarboxylate to ThiS-carboxylate was monitored by ESI-FTMS.

ESI-FTMS Analysis

All protein samples were desalted by binding to a reverse-phase protein trap (Michrom Bioresources, Auburn, CA), followed by washing with MeOH:H_2O:AcOH (1:98:1) and eluting with MeOH:H_2O:AcOH (70:26:4). The resulting protein solutions were electrosprayed at 1 to 50 nl/min with a nanospray emitter. The resulting ions were guided through a heated capillary, skimmer, and three radio frequency-only quadrupoles into a 6 T modified Finnigan FTMS with the Odyssey data system [14].

For MS/MS spectra, specific ions were isolated using stored waveform inverse Fourier-transform (SWIFT), [15] followed by collisionally activated dissociation and IR multiphoton dissociation (IRMPD) [16, 17]. MS/MS spectra were averages of 30–80 scans. Assignments of the fragment masses and compositions were made using the computer program THRASH [18].

Determination of the ^{18}O Content of Regenerated ThiS-Carboxylate

While an approximate ratio of the unlabeled protein versus the labeled protein can be estimated directly from the intensity of the monoisotopic peak and the monoisotopic peak +2 Da, a more accurate estimate was obtained using the program Isopro. The program Isopro, which predicts the distribution of ions in the isotope envelope according to their isotopic composition, was used to generate an isotopic distribution corresponding to the unlabeled ^{13}C- and ^{15}N-depleted ThiS-carboxylate (**A**). This was exported into Excel and a second isotope distribution curve was generated by adding 2 Da to the first distribution (**B**). This represents the isotopic distribution of ThiS-thiocarboxylate containing a single ^{18}O. To get an "estimate" for amount of ^{18}O incorporated into the regenerated ThiS-carboxylate, the observed molecular ion of regenerated ^{13}C-, ^{15}N-depleted ThiS-carboxylate was obtained by changing the ratios of **A** and **B** until the best fit was obtained. This indicated 78% label incorporation from 80% labeled ^{18}O-DXP.

Trapping of Intermediate 14 by $NaBH_4$ Reduction

DXP (125 μM) was added to the solution of ThiS-thiocarboxylate containing ThiG, ThiF, and YrvO, prepared as described above for ThiS-thiocarboxylate (500 μl, 5 mg/ml). The reaction was allowed to proceed for 2–24 hr before reduction at room temperature with 50 mM $NaBH_4$. Excessive foaming was controlled by centrifugation

in a clinical centrifuge. After 20 min, 300 μl of the reaction mixture was buffer exchanged into 20 mM Tris-HCl (pH 7.4), 0.02% NaN_3 using biospin columns (100 μl per column) and stored at −80°C.

Alkylation of the Reduced Intermediate 14

The trapped intermediate (270 μl), prepared as described above, was alkylated with iodoacetamide (30 μl, 100 mM, freshly dissolved in water). After 8 min, 3 × 90 μl of this reaction mixture was added to 3 biospin columns and at exactly 10 min after the addition of the iodoacetamide, the columns were spun to exchange the reaction buffer and excess alkylating agent with 20 mM Tris (pH 7.4), 0.02% NaN_3. The resulting solutions were combined, desalted, and analyzed by ESI-FTMS. ThiS-carboxylate/ThiG and reduced **5** [7] were similarly alkylated and analyzed.

Preparation of [^{35}S]-ThiS-Thiocarboxylate

The reaction mixture consisted of 150 μl of [^{35}S]-cysteine (prepared using 15 μl [227 μCi] of [^{35}S]-cysteine in 150 μl of 2 mM cysteine and 4 mM DTT, in 200 mM Tris [pH 7.8]), 7.5 μl of 200 mM ATP, 7.5 μl of 800 mM $MgCl_2$, 225 μl of ThiS-carboxylate /ThiG/ThiF (40–50 mg/ml, concentrated using a centricon with a 3 kDa cutoff), and 67.5 μl of 2.3 mg/ml YrvO. After 0, 30, 60, and 90 min, 28 μl of this solution was gel filtered into 5 μl of freshly prepared (within one min.) 1000 mM $NaBH_4$. After 20 min, 40 μl of 2× SDS-PAGE buffer was added and the formation of ThiS-thiocarboxylate was verified by SDS-PAGE/autoradiography, and after 90 min, 350 μl of the remaining solution of [^{35}S]-ThiS-thiocarboxylate was buffer exchanged into 200 mM Tris, 4 mM DTT and used for the next experiments.

Trapping of Intermediate 14 for Analysis by SDS-PAGE/Autoradiography

Ten microliters of 25 mM DXP (<1equivalent) was added to the sample of preformed [^{35}S]-ThiS- thiocarboxylate, ThiG, ThiF, and YrvO (350 μl) prepared as described above. Aliquots (28 μl) were removed after 0, 1, 5, 15, and 30 min, and the reaction was quenched by the addition of 5 μl of freshly prepared 1000 mM $NaBH_4$. After 20 min, 40 μl of 2× SDS-PAGE buffer was added to each sample and the resulting solutions were analyzed by SDS-PAGE/autoradiography.

Sulfur Transfer from 14 with or without Glycine

ThiO (10 μl, 12 mg/ml) and glycine (6 μl, 800 mM) were added to 190 μl of **14** prepared as described above. Aliquots (28 μl) were removed after 0, 1, 5, 15, 30, and 60 min. Each aliquot was buffer exchanged into 5 μl of 1000 mM $NaBH_4$ using a biospin column to remove non-protein-bound DXP. After 20 min, 40 μl of 2× SDS-PAGE buffer was added and the sample was analyzed by SDS-PAGE/autoradiography (20 μl for each lane). We loaded only every other lane because the high borate salts caused the bands to spread laterally. As a control, to test that the sulfur transfer from **14** is glycine dependent, a similar reaction was run in the absence of glycine.

Autoradiography of SDS-PAGE Gels

SDS-PAGE gels were washed thoroughly with water and dried in vacuo between gel drying film (Promega) using a BIO-RAD gel dryer (model 583) [5]. The dried gel was exposed to biomax MR (Kodak) autoradiography film for 10 min to 8 days at −80°C, 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. When necessary, the resulting autoradiogram was analyzed by densitometry (Epson expression 1600 with the software U-scan-IT-gel version 3.1).

Assay to Detect Thiazole Phosphate Formation from 14 and Dehydroglycine

A reaction mixture consisting of [^{35}S]-ThiS-thiocarboxylate (30 μl, 1.1 × 10^6 CPM, formed with 35 mg/ml ThiS-carboxylate/ThiG), 3.6 mM HMP, 4 mM ATP, 10 mM $MgCl_2$, 60 μg ThiD, 12 μg ThiE, 8 mM glycine, and 200 μM DXP was preincubated for 20 min before the addition of copurified ThiG/ThiO (150 μg, copurified from 1 liter of ThiG and 0.5 liter ThiO) to bring the total volume to 123 μl. After 2.0 hr at room temperature, 24 nmol of thiamin-phosphate was added as carrier and the reaction was quenched by the addition of 126 μl of 10% TCA. After centrifugation 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 30 s, the reaction was neutralized by the addition of 28 μl of 6 M HCl (pH must be 6.5–7.5). A 100 μl aliquot of this reaction mixture was injected onto a C_{18} HPLC 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 and 4 mM tetrabutylammonium hydrogen sulfate [pH 6.6], and 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; 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 0.9 ml/min. Commercial thiamin monophosphate was oxidized to the thiochrome-phosphate and used as reference with an elution time of 21 min. (Detection: fluorescence excitation at 365 nm and emission at 450 nm, as well as in-line scintillation counting using a Packard flow scintillation analyzer 500TR series.)

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Note Added in Proof

The structure of the thiazole synthase has now been published.

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