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# The Biosynthesis of the Thiazole Phosphate Moiety of Thiamin (Vitamin $B_1$): The Early Steps Catalyzed by Thiazole Synthase

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***Abstract:*** Thiazole synthase (ThiG) catalyzes an Amadori-type rearrangement of 1-deoxy-D-xylulose-5-phosphate (DXP) via an imine intermediate. In support of this, we have demonstrated enzyme-catalyzed exchange of the C2 carbonyl of DXP. Borohydride reduction of the enzyme DXP imine followed by top-down mass spectrometric analysis localized the imine to lysine 96. On the basis of these observations, a new mechanism for the biosynthesis of the thiazole phosphate moiety of thiamin pyrophosphate in *Bacillus subtilis* is proposed. This mechanism involves the generation of a ketone at C3 of DXP by an Amadori-type rearrangement of the imine followed by nucleophillic addition of the sulfur carrier protein (ThiS-thiocarboxylate) to this carbonyl group.

## Introduction

The thiazole moiety of thiamin pyrophosphate (**I**, Figure 1) is biosynthesized in *Bacillus subtilis* from 1-deoxy-D-xylulose-5-phosphate (**VIII**, DXP), glycine, and cysteine in a complex oxidative condensation reaction.[1] This reaction requires five different proteins: Glycine oxidase (ThiO) catalyzes the oxidation of glycine to the corresponding imine (**II** to **III**),[2−5] The adenylyl transferase (ThiF) catalyzes the adenylation of the carboxy terminus of the sulfur carrier protein (**IV**, ThiS), and cysteine desulfurase catalyzes the transfer of sulfur from cysteine to the ThiS acyl adenylate (**V**).[6−8] ThiG is the thiazole synthase and catalyzes the formation of the thiazole from the glycine imine (**III**), DXP (**VIII**), and ThiS-thiocarboxylate (**VII**). It has recently been demonstrated that this system can be greatly simplified: thiazole synthase will catalyze the formation of the thiazole from glyoxylate, ammonia, sulfide, and DXP.[1]

The synthetic chemistry of the thiazole ring has been extensively studied.[9,10] It is therefore surprising that the chemistry used in the biosynthesis of the thiamin thiazole is without chemical precedent. In addition, the biosynthesis of the thiazole moiety of peptide antibiotics, the other major thiazole biosynthetic pathway, which involves the addition of a cysteine thiol to an adjacent amide carbonyl, followed by oxidation, is also different from the biosynthesis of the thiamin thiazole.[11−13]

This paper describes the identification of the early steps involved in the formation of the thiamin thiazole. Thiazole synthase catalyzes H/D exchange at C-3 and oxygen exchange at both C-2 and C-3 of DXP by imine formation with lysine 96 and subsequent Amadori rearrangement. The mechanistic significance of these findings for thiazole formation will be discussed.

## Results

**Thiazole Synthase-Catalyzed H/D Exchange at C-3 of DXP.** When [1-$^{13}$C]-DXP was incubated with thiazole synthase in $D_2O$ and the resulting reaction mixture was analyzed by ESI-MS, the mass of DXP increased from 214 to 215 Da. This mass increase required thiazole synthase and suggested that the enzyme catalyzed the exchange of one of the protons of DXP with solvent. The site of exchange was determined by $^1$H NMR analysis, and a partial spectrum is shown in Figure 2. The top spectrum shows the C3 and the C4 protons of DXP at 4.32 and 4.15 ppm, respectively, for a control reaction run in the absence

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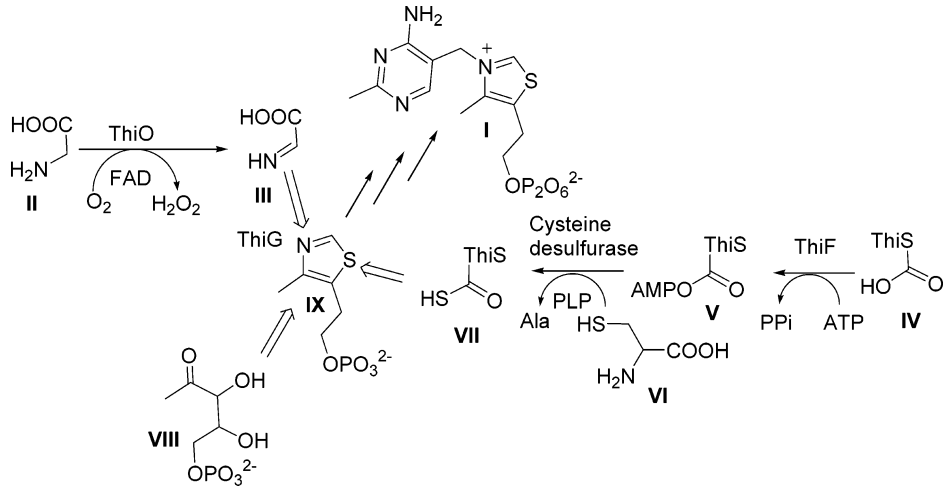


***Figure 1.*** Biosynthesis of thiazole phosphate in *B. subtilis*.

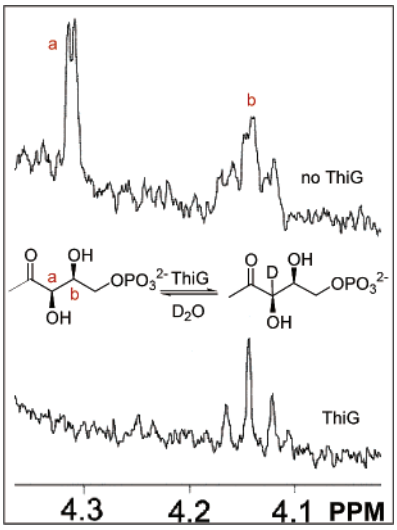


***Figure 2.*** NMR analysis of thiazole synthase-treated DXP. $^1$HNMR of the C-3 (4.32 ppm) and C-4 (4.14 ppm) proton of DXP. Top: DXP incubated in $D_2O$ buffer in the absence of the enzyme. Bottom: DXP incubated in $D_2O$ buffer in the presence of thiazole synthase (ThiG).

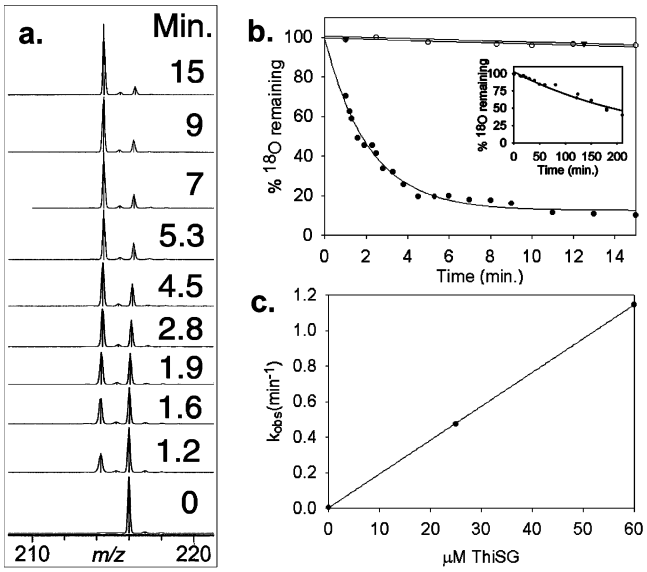


***Figure 3.*** ESI-MS analysis of the thiazole synthase-catalyzed exchange of the C2 carbonyl of DXP. (a) The conversion of [1-$^{13}$C]-[2-$^{18}$O]-DXP (~98% labeled, $m/z$ = 216 Da) to [1-$^{13}$C]-[2-$^{16}$O]-DXP in $^{16}$O-buffer catalyzed by thiazole synthase as observed by ESI-MS. (b) The kinetics of the exchange reaction. ● = enzymatic reaction ($k_{obs}$ = 0.48 min$^{-1}$), ○ = the K96A mutant ($k_{obs}$ = 0.005 min$^{-1}$). ▼ and inset = no enzyme ($k_{obs}$ = 0.005 min$^{-1}$). The curves are fits to a first-order exponential function. (c) The effect of thiazole synthase concentration on the rate of exchange of the C2 carbonyl of DXP.

of enzyme. In this control, no exchange was observed, even after four weeks of exposure to the deuterated buffer. The bottom spectrum is from the sample run in the presence of thiazole synthase and shows that all of the C3 protons have been exchanged; consequently, the signal at 4.32 ppm has disappeared, and the doublet of triplets at 4.14 ppm has collapsed to a triplet.

**Thiazole Synthase-Catalyzed Carbonyl Oxygen Exchange of DXP.** When [1-$^{13}$C, 2-$^{18}$O]-DXP was incubated with thiazole synthase in $H_2O$ and the resulting reaction mixture was analyzed by ESI-MS, the mass of DXP decreased from 216 to 214 Da (Figure 3a). This suggested that thiazole synthase catalyzed the exchange of the carbonyl oxygen of DXP with solvent. The rate of this exchange in the presence of 25 $\mu$M thiazole synthase is 0.48 min$^{-1}$ (Figure 3b). The corresponding rate of exchange in the absence of enzyme or in the presence of the K96A mutant was 0.005 min$^{-1}$. The rate of exchange increased to 1.1 min$^{-1}$ when the WT enzyme concentration was increased to 60 $\mu$M (Figure 3c).

**Thiazole Synthase-Catalyzed Oxygen Exchange of [2,3,4-$^{18}$O]-DXP.** When partially labeled [2,3,4-$^{18}$O]-DXP was incubated with thiazole synthase in $H_2O$ and the resulting reaction mixture was analyzed by ESI-MS, a loss of 4 Da was observed, indicating the exchange of two oxygen atoms. This loss of 4 Da is most easily recognized by comparing the relative concentrations of unlabeled DXP (213 Da) with those of the singly labeled DXP (215 Da) in Figure 4. If only one oxygen was exchanged, the maximum level of unlabeled DXP would have been 15% (sum of the 213 and 215 Da species). Since we observe 31% of unlabeled DXP after incubation for 90 min., the additional 16% had to be formed by the exchange of two oxygen atoms from doubly labeled DXP.

**Trapping of an Imine between Thiazole Synthase and DXP.** When a reaction mixture containing thiazole synthase and [1-$^{13}$C]-DXP was reduced with $NaBH_4$ and analyzed by ESI-FTMS, a new protein species was detected, in addition to

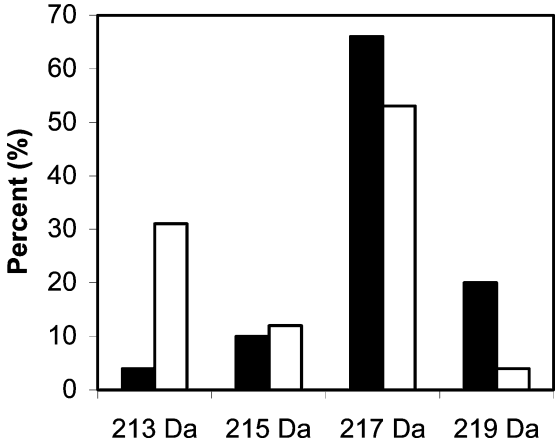


***Figure 4.*** Thiazole synthase-catalyzed exchange of [2,3,4-$^{18}$O]-DXP. Black bar: isotope distribution of the partially labeled [2,3,4-$^{18}$O]-DXP. White bar: isotope distribution in partially labeled [2,3,4-$^{18}$O]-DXP after treatment with thiazole synthase.

thiazole synthase (Figure 5, parts a and b). This new species (27 004.0 Da) has a mass that is 200.1 Da heavier than thiazole synthase (26 803.9 Da). We expect a mass increase of 200.04 Da for the reduction of the imine between [1-$^{13}$C]-DXP and thiazole synthase.

**Localization of the Imine between Thiazole Synthase and DXP.** The 27 004.0 Da molecular ion was SWIFT isolated and fragmented using IR multiphoton dissociation (IRMPD), sustained off-resonance irradiation dissociation (SORI−CAD) and electron capture dissociation (ECD) to give 117 unique interresidue cleavages and to produce the fragmentation map shown in Figure 5d. Fragments carrying the 200.1 Da adduct are indicated with an asterisk in the fragmentation map. The region to which the 200.1 Da adduct is localized is K96−R104. Since this fragment contains only one lysine residue, the imine must have been formed between K96 and DXP. In support of this, K96 is an absolutely conserved thiazole synthase residue and the K96A mutant does not form an adduct with DXP (Figure 5c). In addition, the K96A thiazole synthase is unable to form thiazole phosphate as detected using the thiochrome assay (data not shown).[1]

## Discussion

Alignment of thiazole synthase (ThiG) sequences that cluster with other thiazole biosynthetic genes[14] from phylogenetically diverse microorganisms indicated that lysine 96 (*B. subtilis* numbering[15]) is absolutely conserved. This observation suggested that this lysine residue might form an imine with the carbonyl group of DXP.

Imine formation is a widely used strategy in enzymology to increase the acidity of a proton alpha to a carbonyl group, and model studies have shown that imine deprotonation at the α-carbon is $10^8$ times faster than the corresponding carbonyl deprotonation.[16,17] Imine intermediates are used in the catalysis of many aldol condensation reactions and decarboxylation reactions.[18−34] Mechanistic evidence for an imine intermediate includes the demonstration of enzyme-catalyzed H/D exchange alpha to the carbonyl group,[23,34] the identification of an essential conserved lysine,[21,27] the demonstration of enzyme-catalyzed exchange of the carbonyl group oxygen,[18,32−34] the trapping of the imine by borohydride reduction to give a single covalent adduct,[19,20,22,24,25,33] and the localization of the adduct to the conserved lysine residue.[25] We have applied all five of these criteria to probe for a thiazole synthase/DXP imine intermediate.

Treatment of [$^{18}$O]-DXP with thiazole synthase in $H_2O$ containing buffer resulted in exchange of the carbonyl oxygen. This exchange was 230 times faster than the nonenzymatic exchange using 60 μM enzyme. (Figure 3). The K96A mutant of thiazole synthase did not catalyze the exchange. In addition, we have demonstrated that thiazole synthase can catalyze the exchange of two of the oxygen atoms from partially labeled [2,3,4-$^{18}$O]-DXP (Figure 4).

To identify the lysine residue involved in imine formation, we incubated thiazole synthase with DXP and reduced with $NaBH_4$ to trap the putative imine. ESI-FTMS analysis of this sample revealed the formation of a single new species with a mass of 27 004.0 Da (Figure 5b). This was consistent with the reduced thiazole synthase/DXP imine complex. MS analysis of this species localized the adduct to the K96−R104 peptide, which contains only a single lysine residue (K96). This confirmed our prediction that K96 forms an imine with DXP. In further support of this, the K96A mutant of thiazole synthase (57 Da lighter than the native enzyme) does not form an adduct with DXP (Figure 5c) and does not catalyze the carbonyl oxygen exchange reaction. To eliminate the possibility that the DXP/thiazole synthase imine is an artifact, we have also demonstrated that the K96A mutant is not able to catalyze the formation of thiazole phosphate in our in vitro reconstitution system (data not shown).[1]

Thiazole synthase also catalyzes the exchange of the C3 proton of DXP with solvent (Figure 2) by forming an imine with C2. This exchange at C3 is at least 1000 times faster than the buffer-catalyzed exchange.

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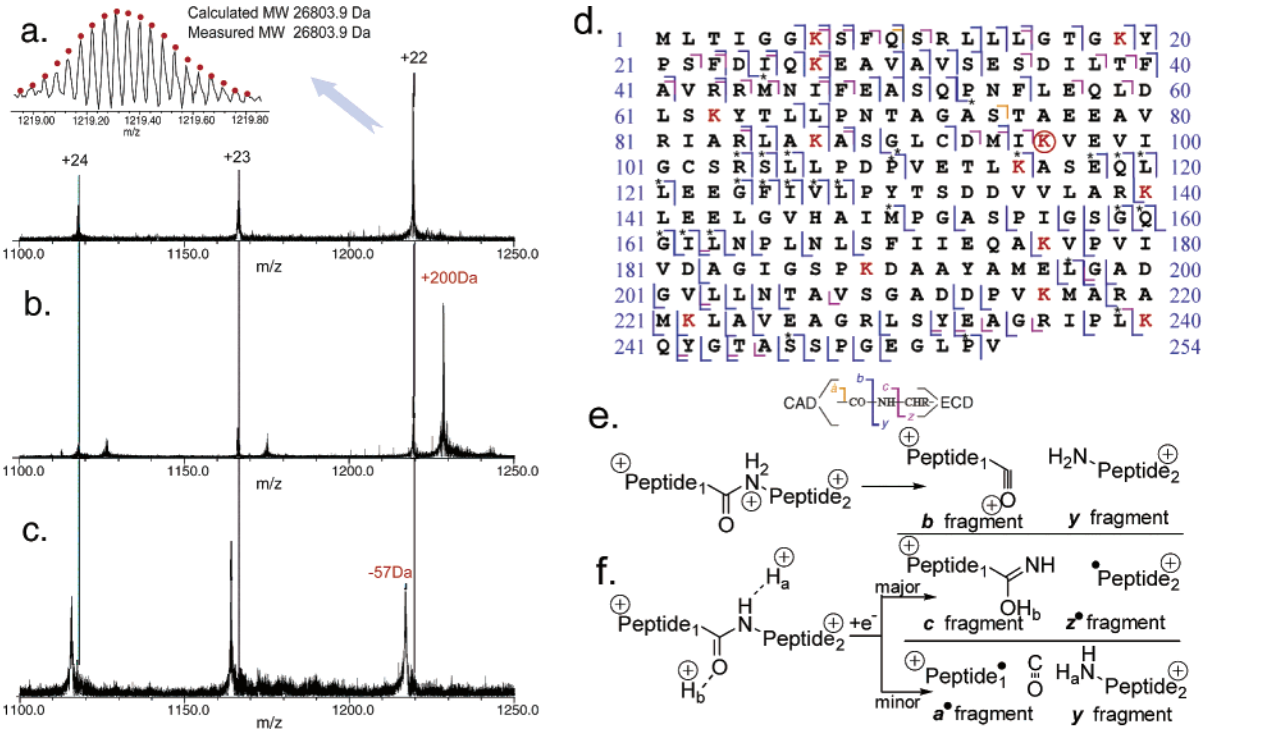


***Figure 5.*** Localization of the [1-$^{13}$C]-DXP-thiazole synthase imine by $NaBH_4$ reduction followed by top-down ESI-FTMS analysis. (a) ESI-FTMS of three different charge states of thiazole synthase. Inset: enlargement of the spectrum of thiazole synthase with the theoretical molecular weight simulated by the dots. (b) DXP and thiazole synthase reduced with $NaBH_4$. (c) DXP and thiazole synthase (K96A) reduced with $NaBH_4$. (d) The localization of the covalently modified residues by MS-MS. Observed thiazole synthase fragments that are covalently modified with DXP are denoted with *. For example, $^{*}]$ indicates a labeled b ion. The labeled fragments extend from the site of cleavage to either the amino or the carboxy terminus of the protein. (e) Fragmentations observed with IRMPD and SORI-CAD. (f) Fragmentations observed with ECD.

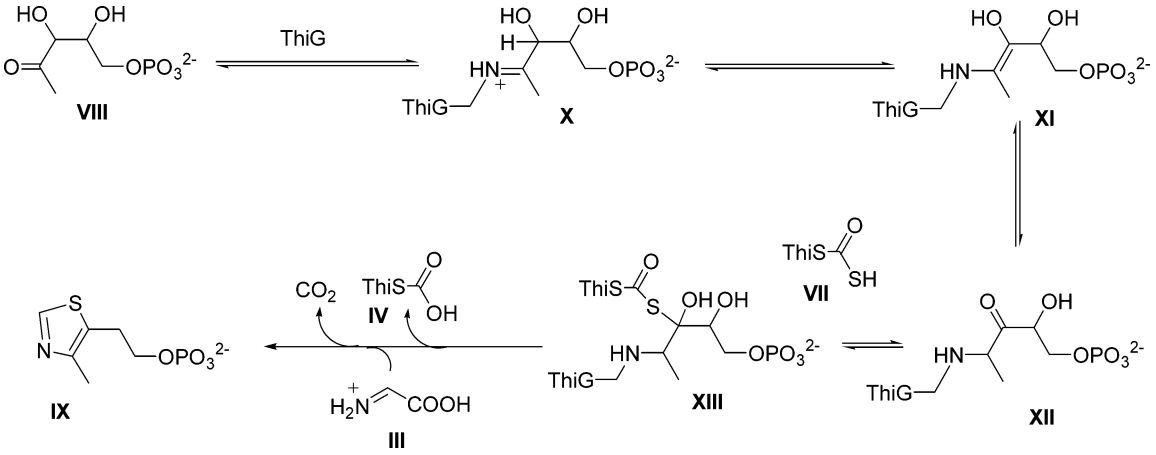


***Figure 6.*** Mechanistic proposal for the thiazole synthase-catalyzed formation of thiazole phosphate in *B. subtilis.*

A mechanistic proposal for the formation of the thiazole phosphate moiety of thiamin pyrophosphate is outlined in Figure 6. In this proposal, DXP forms an imine with lysine 96 of thiazole synthase, and deprotonation at C3 followed by protonation at C2 gives **XII**. The first carbon sulfur bond of the thiazole could then be formed by addition of the ThiS thiocarboxylate to the C3 carbonyl to give **XIII**. This proposal is consistent with the identification of the K96/DXP imine, with the observed carbonyl oxygen exchange as well as the H/D exchange at C3. This proposal is also consistent with the observed exchange of two oxygen atoms from DXP, which could occur by water addition to the carbonyl group of **XII**. Several mechanisms for the conversion of **XIII** to the thiazole phosphate (**IX**) can be envisioned, and experiments are currently underway to establish the mechanistic details of the middle and late steps of thiazole formation.

## Experimental Section

**Materials.** NTA resin was the NTA superflow by Qiagen. $D_2O$, pyruvate, fructose bis-phosphate, aldolase, triosephosphate isomerase, and thiamin pyrophosphate were all purchased from Sigma-Aldrich. TLC plates were Kieselgel 60 $F_{254}$ produced by Merck. The microcon membrane filters were from Millipore.

**General.** DXP and [1-$^{13}$C]-DXP were synthesized as described.[35,36] 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. The purity of all the proteins was assessed by 12% (DXP synthase, ThiG) or 16% (ThiSG and ThiSG(K96A)) SDS-PAGE. The proteins were quantitated using the Bradford assay using BSA as standard. ESI-MS

analysis of DXP, [1-$^{13}$C]-DXP, [1-$^{13}$C]-[2-$^{18}$O]-DXP, and [2,3,4-$^{18}$O]-DXP was performed using an Esquire instrument (Bruker). For this analysis, we observed the region of *m*/*z* 180−250 Da in a negative ion mode. Typical settings for the spectrometer: flow rate = 120−240 $\mu$l/min, skim voltage = −19.8 V, cap exit = −89.0 V, and trap drive = 35.0 V, and 8 scans were collected. If the signal was of poor quality, the region from 204 to 224 was isolated using the quadrupole, which enhanced the signal.

**Protein Overexpression and Purification.** *B. subtilis* thiazole synthase (ThiG, for stability it is often co-purified with ThiS) was overexpressed and purified as previously described.[1] For the overexpression of DXP synthase, *Escherichia coli* BL21(DE3) containing the plasmid pCLK1032 (pET16b derived) was grown in LB medium (containing 40 mg/L ampicillin) to an OD of 0.6, at which point it was induced with 100 mg/L of IPTG, and the cells were grown for an additional 6 h. The cells were harvested by centrifugation, and the resulting cell paste was stored at −80 °C until further use. To isolate the enzyme, the cell pellet was thawed and lysed in 10 mL of lysis buffer (100 $\mu$M thiamin pyrophosphate, 50 mM KPi, 300 mM NaCl, 10 mM imidazole, pH 8.2) by the addition of 5 mg of lysozyme and two 20 s cycles of sonication (50% power). The resulting cell extract was clarified by centrifugation and loaded onto an NTA column (2.5 mL of Qiagen superflow resin in a 1.5-cm diameter column) which was preequilibrated with lysis buffer. The NTA column was washed with 5 mL of lysis buffer followed by 15 mL of wash buffer (100 $\mu$M thiamin pyrophosphate, 50 mM KPi, 300 mM NaCl, 30 mM imidazole, pH 8.2). The protein was eluted with elution buffer (100 $\mu$M thiamin pyrophosphate, 50 mM KPi, 300 mM NaCl, 250 mM imidazole, pH 8.2), and 0.5 mL fractions were collected. The most concentrated fractions, as determined by the Bradford assay or the absorbance at 280 nm, were buffer-exchanged into 50 mM Tris-HCl, 100 $\mu$M thiamin pyrophosphate, pH 7.8 using a PD-10 gel filtration column (Amersham Biosciences) and stored at −80 °C in 2−5% glycerol. The activity of this protein was determined by TLC analysis (6:1:3 *N*-propanol/ethyl actetate/water, Rf 0.6) of a reaction mixture containing fructose-1,6-bis-phosphate, aldolase, triose-phosphate isomerase, pyruvate, and DXP-synthase.[35−38] DXP was visualized by dipping the plate into a ceric ammonium molybdenate solution and heating the plate to 300 °C for 30−60 s until a blue spot appeared. $^1$H NMR indicated the presence of DXP (400 MHz, $D_2O$, ppm): 4.3 (s, 1H), 4.1 (t, 1H), 3.6 (dd, 2H), 2.1(s, 3H). ESI-MS (negative ion mode): 213 Da. For unknown reasons, only one out of 10 preparations of DXPS yielded active protein; once active, however, the protein was stable for months as a glycerol stock at −80 °C.

**Reduction of the Thiazole Synthase/DXP Complex with $NaBH_4$.** A typical experiment was carried out by incubating [1-$^{13}$C]-DXP (250 $\mu$M) in 500 $\mu$l of 40 mM Tris-HCl, pH 7.7 containing thiazole synthase (10 mg/mL, as ThiSG) for 2 h. The reaction mixture was then reduced by treatment with $NaBH_4$ (200 mM) for 7−10 min. The foam generated during the reduction was spun down in a clinical centrifuge for 2 min, and 200 $\mu$L of this solution was gel-filtered using multiple biospin columns (BIO-RAD Tris, pH 7.4, 0.02% $NaN_3$) to remove excess substrate and boron salts. The resulting protein solution (200 $\mu$L) was frozen and stored at − 80 °C until further use.

**ESI-FTMS Analysis and Localization of the Modified Residue.** The samples prepared above were desalted using reverse-phase protein traps (Michrom Bioresources, Auburn, CA), washed with MeOH/$H_2O$/AcOH (1:98:1), and eluted with MeOH/$H_2O$/AcOH (70:26:4). Protein solutions were electrosprayed at 1−50 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 outfitted with the Odyssey data acquisition and analysis system. For MS/MS spectra, specific ions were isolated using stored waveform inverse Fourier transform (SWIFT),[39] followed by collisionally activated dissociation (SORI-CAD), IR multiphoton dissociation (IRMPD),[40] as well as electron capture dissociation (ECD).[41] MS/MS spectra were averages of 30−80 scans. Assignments of the fragment masses and compositions were made with the computer program THRASH.[42]

**Preparation of [1-$^{13}$C]-[2-$^{18}$O]-DXP.** To exchange the C-2 carbonyl on DXP, 10.0 $\mu$L of a stock solution of [1-$^{13}$C]-DXP (25 mM) was lyophilized and then redissolved in 5.0 $\mu$L of 98% $H_2{}^{18}O$. After standing for 12−24 h at room temperature, the sample was stored at −80°C until further use. ESI-MS (negative ion mode): 216 Da.

**Thiazole Synthase-Catalyzed Exchange of [2-$^{18}$O]-DXP.** Purified thiazole synthase (as ThiSG) was buffer-exchanged into 1 mM Tris-HCl (pH 7.8), and 250 $\mu$L of this protein solution (2.4 mg/mL) was added to the 5 $\mu$L of [1-$^{13}$C]-[2-$^{18}$O]-DXP described above to give a final concentration of 1 mM DXP. The conversion of [1-$^{13}$C]-[2-$^{18}$O]-DXP to [1-$^{13}$C]-[2-$^{16}$O]-DXP was directly monitored by ESI-MS in the negative ion mode. A spectrum was saved every 0.5−2 min (the exact time was recorded) over 15 min. To calculate the conversion rates, the relative intensities of the 216 Da signal ([1-$^{13}$C]-[2-$^{18}$O]-DXP) and the 214 Da ([1-$^{13}$C]-[2-$^{16}$O]-DXP) signal were plotted against time and fitted to a single-exponential function using Sigmaplot. The K96A thiazole synthase reaction and the nonenzymatic reaction were run and analyzed in a similar fashion.

**Thiazole Synthase-Catalyzed H/D Exchange of DXP Analyzed by ESI-MS.** To observe the H/D exchange of DXP by ESI-MS, 2 mL of 100 mM Tris-HCl was lyophilized (pH 7.6) and redissolved in 40 mL of $D_2O$. Purified thiazole synthase (purified as ThiSG, 5 mg/mL) was buffer-exchanged into this buffer by gel filtration. [1-$^{13}$C]-DXP (1 mM) was added to this protein solution (500 $\mu$L). After 6 h, the reaction mixture was filtered through a 3 kDa membrane (microcon, 6000 rpm). The flow-through was lyophilized, redissolved in water/2-propanol (1:1) and analyzed by ESI-MS. A similar reaction, from which thiazole synthase was omitted, was run as a control.

**Thiazole Synthase-Catalyzed H/D Exchange of DXP Analyzed by $^1$HNMR.** To observe the H/D exchange of DXP by NMR, 2 mL of 400 KPi (pH 7.6) was lyophilized and redissolved in 40 mL of $D_2O$. Purified thiazole synthase (purified as ThiSG) was buffer-exchanged into this buffer by gel filtration. [1-$^{13}$C]-DXP (1 mM) was added to this protein solution (500 $\mu$L). After 6 h, the reaction mixture was filtered through a 3 kDa membrane (microcon, 6000 rpm), and the membrane was washed with $D_2O$ (600 $\mu$L) to ensure that most of the DXP had passed through the membrane. The flow-through was lyophilized, redissolved in 600 $\mu$L $D_2O$, and analyzed by $^1$HNMR. A similar reaction from which thiazole synthase was omitted was run as control.

**Synthesis of [2,3,4-$^{18}$O]-DXP.**[43] A solution (200 $\mu$L) containing 20 mM dihydroxyacetone-phosphate, 50 mM Tris, and 2 mM DTT (pH 7.8) was lyophilized and redissolved in 80 $\mu$L [$^{18}$O]-$H_2O$ containing 200 units of triose-phosphate isomerase. After 6 h at room temperature, pyruvate (20 mM), thiamin pyrophosphate (20 $\mu$M), and DXP-synthase (20 $\mu$L, 2 mg/mL) were added. After 24 h at room temperature, the

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proteins were removed by membrane filtration (3 kDa cutoff), and the resulting solution of partially labeled [2,3,4-$^{18}$O]-DXP was frozen at −80 °C in 10 $\mu$L aliquots until use. TLC analysis (6:1:3 *N*-propanol/ethyl actetate/water, Rf) 0.6 (ESI-MS, $M^-$): 213, 215, 217, and 219 Da.

A larger sample was prepared for analytical purposes to confirm that the enzymatic synthesis was working. This reaction was run in $H_2O$. $^1$H NMR indicated that DXP had formed (400 MHz, $D_2O$, ppm): 4.3 (s, 1H), 4.1 (t, 1H), 3.6 (t, 2H), 2.1 (s, 3H) and ESI-MS (negative ion mode): 213 Da.

**Thiazole-Catalyzed Exchange of [2,3,4 $^{18}$O]-DXP.** [2,3,4-$^{18}$O]-DXP (10 $\mu$L) was diluted with 190 $\mu$L of thiazole synthase (ThiSG, 5 mg/mL) in 1 mM Tris HCl, pH 7.8 and directly analyzed by ESI-MS over 90 min. The observed molecular ions at 213, 215, 217, and 219 *m*/*z* were normalized to 100%.

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