Mechanistic Studies on Thiamin Phosphate Synthase: Evidence for a Dissociative Mechanism†

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ABSTRACT: Thiamin phosphate synthase catalyzes the coupling of 4-methyl-5-(β-hydroxyethyl)thiazole phosphate (Thz-P) and 4-amino-5-(hydroxymethyl)-2-methylpyrimidine pyrophosphate (HMP-PP) to give thiamin phosphate. In this paper, we demonstrate that 4-amino-5-(hydroxymethyl)-2-(trifluoromethyl)-pyrimidine pyrophosphate (CF_3-HMP-PP) is a very poor substrate [$k_{cat}(CH_3) > 7800k_{cat}(CF_3)$] and that 4-amino-5-(hydroxymethyl)-2-methoxypyrimidine pyrophosphate (CH_3O-HMP-PP) is a good substrate [$k_{cat}(OCH_3) > 2.8k_{cat}(CH_3)$] for the enzyme. We also demonstrate that the enzyme catalyzes positional isotope exchange. These observations are consistent with a dissociative mechanism (S_N1 like) for thiamin phosphate synthase in which the pyrimidine pyrophosphate dissociates to give a reactive pyrimidine intermediate which is then trapped by the thiazole moiety.

Thiamin phosphate synthase catalyzes the formation of thiamin phosphate **3** from 4-amino-5-(hydroxymethyl)-2-methylpyrimidine pyrophosphate (**1**) and 4-methyl-5-(β-hydroxyethyl)thiazole phosphate (**2**) (Figure 1). This reaction is the penultimate step in the biosynthesis of thiamin pyrophosphate. The gene for this enzyme has been cloned and overexpressed from *Escherichia coli* (*1*, *2*), *Bacillus subtilis* (*3*), *Saccharomyces cerevisiae* (*4*), and *Arabodopsis thaliana* (*5*), and orthologs can be identified in all thiamin-synthesizing organisms where sequence information is available. The structure of the enzyme from *B. subtilis*, with thiamin phosphate and pyrophosphate bound at the active site, has recently been reported (*6*).

Thiamin phosphate formation could occur by an associative or a dissociative displacement mechanism (Figure 2). In this paper, we describe the use of substituent effects and a positional isotope exchange experiment to differentiate between these two possibilities.

MATERIALS AND METHODS

4-Amino-5-(chloromethyl)-2-methylpyrimidine (*7*), 4-amino-5-(aminomethyl)-2-methylpyrimidine (Grewe diamine) (*8*), and 3-chloro-4-oxopentyl acetate (*9*) were provided as gifts by Hoffman-La Roche. Thiamin phosphate synthase and HMP-P[1] kinase were overexpressed and purified as previously described (*3*, *10*). THF was dried and distilled from potassium/benzophenone. Methylene chloride was dried and distilled from calcium hydride. All other organic reagents were obtained from Aldrich, and used without further purification. Bovine intestinal mucosal alkaline phosphatase and Type IV-S potato acid phosphatase were obtained from Sigma. $H_2{}^{18}O$ was purchased from Cambridge Isotope Laboratories. Flash chromatography was performed with EM Science silica gel 60 (230−400 mesh). NMR spectra were recorded on a Varian 200 MHz or Varian INOVA 400 MHz instrument. ESI mass spectrometry was performed with a Micromass Quattro 1 triple-quadrupole tandem mass spectrometer. δ values are given in parts per million.

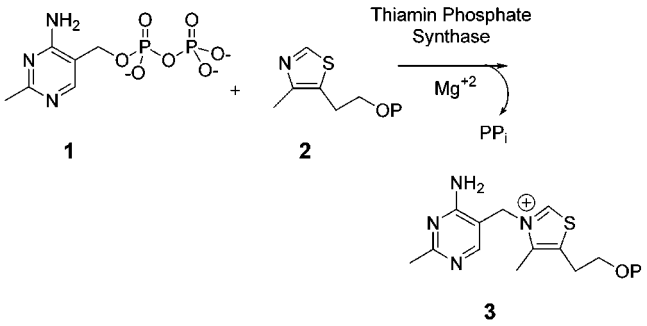

FIGURE 1: Reaction catalyzed by thiamin phosphate synthase.

*4-Amino-5-(hydroxymethyl)-2-methylpyrimidine (HMP, **5**).* A solution containing 389 mg (5.64 mmol) of $NaNO_2$ in 5 mL of water was slowly added, using a dropping funnel, to a solution of 1.08 g (5.10 mmol) of Grewe diamine• dihydrochloride in 12.5 mL of 10% HCl, at 90 °C. The reaction mixture was immediately cooled, titrated to pH 8.5 with 50% NaOH, and concentrated in vacuo to give a yellow residue which was triturated several times with anhydrous methanol. The methanol was removed in vacuo, and the

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[1] Abbreviations: Thz-P, 4-methyl-5-(β-hydroxyethyl)thiazole phosphate; HMP-PP, 4-amino-5-(hydroxymethyl)-2-methylpyrimidine pyrophosphate; CF_3-HMP-PP, 4-amino-5-(hydroxymethyl)-2-(trifluoromethyl)pyrimidine pyrophosphate; CH_3O-HMP-PP, 4-amino-5-(hydroxymethyl)-2-methoxypyrimidine pyrophosphate; HMP-P, 4-amino-5-(hydroxymethyl)-2-methylpyrimidine phosphate; THF, tetrahydrofuran; HMP, 4-amino-5-(hydroxymethyl)-2-methylpyrimidine; CF_3-HMP, 4-amino-5-(hydroxymethyl)-2-(trifluoromethyl)pyrimidine; MeO-HMP, 4-amino-5-(hydroxymethyl)-2-methoxypyrimidine; PIX, positional isotope exchange; TPS, thiamin phosphate synthase.

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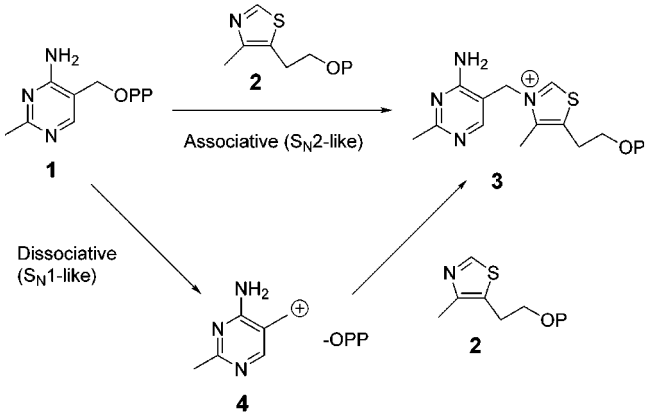


FIGURE 2: Two mechanistic proposals for thiamin phosphate synthase.

residue was purified by flash chromatography (silica gel, 90:10 chloroform/methanol mixture) to yield 524 mg (3.8 mmol, 74%) of HMP as a colorless solid: ^{1}H NMR (200 MHz, D_2O) δ 7.99 (s, 1H), 4.52 (s, 2H), 2.39 (s, 3H).

*4-Amino-5-(hydroxymethyl)-2-methylpyrimidine Pyrophosphate (HMP-PP, **1**).* A 0.2 mM stock solution of HMP-PP was prepared by adding 40 μL of a HMP-P kinase solution (16 mg/mL) to a 1 mL solution containing 0.2 mM HMP and 5 mM ATP in reaction buffer [6 mM $MgCl_2$ and 100 mM triethanolamine·HCl (pH 8)]. The enzymatic reaction went to completion at room temperature in 30 min as demonstrated by the formation of 0.4 mM ADP, which was assayed as previously described (*10*). The HMP-PP generated in this manner was used without further purification because ADP, ATP, and HMP-P kinase did not inhibit the thiamin phosphate synthase reaction.

*4-Amino-5-(hydroxymethyl)-2-(trifluoromethyl)pyrimidine Pyrophosphate (CF_3-HMP-PP, **8**).* CF_3-HMP (**6**) (*11*) (0.2 mM) was pyrophosphorylated using HMP-P kinase as described above for the preparation of HMP-PP.

*4-Amino-5-(hydroxymethyl)-2-methoxypyrimidine Pyrophosphate (MeO-HMP-PP, **9**).* MeO-HMP (**7**) (*12*) (0.2 mM) was pyrophosphorylated using HMP-P kinase as described above for the preparation of HMP-PP.

*4-Amino-5-(chloromethyl)-2-(trifluoromethyl)pyrimidine (**15**).* This compound was prepared by a modification of the literature procedure (*13*). A mixture of 4-amino-5-(hydroxymethyl)-2-(trifluoromethyl)pyrimidine (150 mg, 0.78 mmol), thionyl chloride (3 mL, 4.9 g, 41 mmol), and 7.5 mL of chloroform was refluxed with stirring for 7 h. The mixture was concentrated in vacuo and purified by flash chromatography (2:1 hexanes/ethyl acetate mixture) to yield 136 mg of white solid (0.64 mmol, 82%): ^{1}H NMR (200 MHz, d_6-acetone) δ 8.4 (s, 1H), 7.0 (br, 2H), 4.8 (s, 2H).

*2′-(Trifluoromethyl)thiamin (**16**).* This compound was prepared by a modification of the literature procedure (*13*). A mixture of 4-amino-5-(chloromethyl)-2-(trifluoromethyl)-pyrimidine, **15** (52.7 mg, 0.25 mmol), and 4-methyl-5-(β-hydroxyethyl)thiazole (53.5 mg, 0.37 mmol) was heated in a 145−150 °C oil bath as a melt for 13 min. After cooling, the reaction mixture was dissolved in hot absolute ethanol and acidified with freshly prepared ethanolic HCl, and the solvent was removed in vacuo. The crystallinity of the sample was improved by repeating this procedure twice. The precipitate resulting from the third addition of ethanolic HCl was filtered, and the white solid was washed with ethanolic HCl. The product was recovered from the filter by dissolving it in hot ethanol followed by solvent removal in vacuo to yield 49.2 mg (0.13 mmol, 50%) of 2′-(trifluoromethyl)-thiamin hydrochloride as a white solid: TLC (silica gel, 6:3:1 ethyl acetate/methanol/ammonium hydroxide) $R_f \sim 0.15$; ^{1}H NMR (200 MHz, DMSO) δ 10.19 (s, 1H), 8.74 (s, 1H), 8.46 (br, 2H), 5.88 (s, 2H), 4.15 (t, 2H), 3.55 (t, 2H), 2.99 (s, 3H).

*4-Amino-5-(azidomethyl)-2-methoxypyrimidine (**10**).* Diphenyl phosphroryl azide (0.18 mL, 0.86 mmol) was added to a suspension of 4-amino-5-(hydroxymethyl)-2-methoxypyrimidine, **7** (*12*) (111 mg, 0.72 mmol), in 13 mL of THF. 1,8-Diazabicyclo[5.4.0]undec-7-ene (0.93 mmol, 142 mg) was immediately added to the resulting reaction mixture. After the mixture had been kept at room temperature for 30 min, the reaction was quenched by adding water (7 mL) and the mixture extracted with ethyl acetate (4 × 15 mL). The organic phase was dried (Na_2SO_4), and the solvent was removed in vacuo. The product (78.4 mg, 0.44 mmol, 61% yield) was purified by flash chromatography (silica gel, 3:1 ethyl acetate/hexanes mixture) to give a white solid: ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.98 (s, 1H), 5.23 (br, 2H), 4.21 (s, 2H), 3.91 (s, 3H).

*4-Amino-5-(aminomethyl)-2-methoxypyrimidine (**11**).* Hydrogen gas was bubbled vigorously through a solution of 4-amino-5-(azidomethyl)-2-methoxypyrimidine, **10** (78.4 mg, 0.44 mmol), in ethanol (10 mL) containing 80 mg of 10% Pd/C. After 15 min, the catalyst was removed by filtration and the solvent was evaporated to give the product (61 mg, 0.4 mmol, 91% yield) as the free amine, which was used in the next step without further purification: ^{1}H NMR (200 MHz, DMSO) δ 7.78 (s, 1H), 6.86 (br, 2H), 3.78 (s, 3H), 3.52 (s, 2H), 3.25 (br, 2H).

*2′-Methoxythiamin (**14**).* 2′-Methoxythiamin was prepared from **11** using modifications to a route previously developed for the synthesis of thiamin (*14*). To 109 mg (0.71 mmol) of 4-amino-5-(aminomethyl)-2-methoxypyrimidine were added the following in order: 1.2 mL of 75% ethanol (aqueous), 0.11 mL of 20% ammonium hydroxide, 189 mg (1.06 mmol) of 3-chloro-4-oxopentyl acetate (*9*), 0.28 mL of water, and 70 mg (0.92 mmol) of carbon disulfide. The mixture was stirred at room temperature overnight. Water (1 mL) was added, and the product was extracted several times into ethyl acetate. The organic layer was dried over sodium sulfate, and the solvent was removed in vacuo. The product was partially purified by flash chromatography (10% methanol in chloroform) to yield 90 mg of **12**. This was heated in 10% aqueous HCl (1 mL) in a 90−95 °C oil bath for 15 min. The mixture was allowed to cool to room temperature and was neutralized with 10% NaOH (~1 mL) to precipitate **13** as a white solid. This was dissolved in 10% HCl (0.6 mL) and cooled in an ice bath. Hydrogen peroxide (83 μL, 30%) was added; the reaction mixture was stirred at room temperature for 30 min, and the product was precipitated by the addition of 3−5 mL of saturated aqueous barium chloride. This precipitate was collected by filtration. The crude solid was purified by suspending it in a small volume of diethyl ether, heating to boiling, and adding ethanol until the solid dissolved. The flask was then capped and allowed to slowly cool to room temperature. The resulting white solid was collected by filtration to yield 20 mg of 2′-methoxythiamin

14 as the hydrogen chloride salt (8% yield overall from **10**): ^{1}H NMR (200 MHz, D_2O) δ 9.48 (s, 1H, exchanges in D_2O), 7.98 (s, 1H), 5.42 (s, 2H), 4.05 (s, 3H), 3.85 (t, 2H), 3.25 (t, 2H), 2.55 (s, 3H).

*[^{18}O]-4-Amino-5-(hydroxymethyl)-2-methylpyrimidine ([^{18}O]-HMP, **18**).* A solution of 4-amino-5-(chloromethyl)-2-methylpyrimidine, **17** (850 mg, 5.4 mmol), in $H_2{}^{18}O$ (1 mL) was heated at 50 °C in a sealed tube for 18 h. Sodium azide (350 mg, 5.4 mmol) was then added, and the reaction mixture was stirred for an additional 30 min. Solvent was removed in vacuo. Column chromatography (silica gel, 90:10 methylene chloride/methanol mixture) yielded [^{18}O]HMP as a white solid (578 mg, 76%): ^{1}H NMR (200 MHz, D_2O) δ 7.99 (s, 1H), 4.52 (s, 2H), 2.39 (s, 3H).

*[^{18}O]-4-Amino-5-(hydroxymethyl)-2-methylpyrimidine Pyrophosphate (**19**).* A 1 mL solution containing 2 mM [^{18}O]-HMP, 5 mM ATP in reaction buffer [6 mM $MgCl_2$ and 100 mM triethanolamine·HCl (pH 8)], and 0.6 mg of HMP-P kinase was incubated at room temperature for 3 h. The resulting crude sample was used directly for the PIX experiment. This compound was not purified because it undergoes nonenzymatic PIX during purification. Since the HMP is at substrate inhibition concentrations for HMP-P kinase, the reaction did not go to completion. HPLC analysis (*10*) indicated that this enzyme reaction mixture contained 51% HMP-PP and 49% HMP-P.

*Potassium Diethyl Acetonedicarboxylate (**21**) (15).* Diethyl 1,3-acetone dicarboxylate, **20** (10 g, 49.5 mmol), was added to a solution of potassium (1.93 g, 49.5 mmol) in 100 mL of absolute ethanol. After 15 min, the solvent was removed in vacuo to yield a fluffy greenish-white solid that was used without further purification.

α*-Bromodicarbethoxyacetone (**22**) (15).* Bromine (2.35 mL, 45.6 mmol) was slowly added using a syringe to a suspension of potassium diethyl acetonedicarboxylate, **21** (11.2 g, 46.6 mmol), in 140 mL of dry chloroform. After 30 min, the reaction mixture was filtered and the solvent was removed in vacuo. The crude product was partially purified by quickly filtering it through a 1 in. plug of silica, using a 3:2 hexanes/ethyl acetate mixture, and the solvent was removed to give the product as an orange oil (11.4 g, 35.7 mmol, 77% yield). This mixture was used for the synthesis of **23** without further purification: ^{1}H NMR δ 5.0 (s, 1H), 4.4−4.0 (two overlapping quartets, 4H), 3.8 (s, 2H), 1.4−1.25 (two overlapping triplets, 6H).

*2-Amino-4-(carbethoxymethyl)-5-carbethoxythiazole (**23**) (15).* A mixture of bromide **22** (11.4 g, 35.7 mmol) and thiourea (2.99 g, 39.3 mmol) in absolute ethanol (140 mL) was heated at reflux for 10 min. Most of the solvent was then removed by distillation. The product was precipitated by the addition of ∼10 mL of 5% NaOH followed by cooling at 4 °C overnight. The product was removed by filtration and purified by flash chromatography (silica, 98:2 methylene chloride/methanol mixture) to yield **23** as a white solid (5.68 g, 22 mmol, 62% yield): ^{1}H NMR δ 5.9 (broad, 2H), 4.3−4.1 (two overlapping quartets, 4H), 4.0 (s, 3H), 1.4−1.2 (two overlapping triplets, 6H).

*2-Amino-4-(β-hydroxyethyl)-5-methylthiazole (**24**) (15).* A solution of thiazole **23** (4.34 g, 16.8 mmol) in 500 mL of anhydrous ether was added slowly using a dropping funnel to a stirred suspension of lithium aluminum hydride (5.55 g, 146 mmol) in ether (250 mL). The reaction mixture was stirred under argon at room temperature for 24 h; the reaction was quenched by the addition of water (24 mL), and the mixture was stirred for 2 h and then filtered. The precipitate was washed with ether. The washings were combined with the filtrate, and the solvent was removed in vacuo to yield crude **24** as an oil (1.92 g, 12.1 mmol, 72% yield). This was used without further purification for the next step: ^{1}H NMR δ 3.84 (t, 2H), 2.66 (t, 2H), 2.19 (s, 3H).

*5-Methyl-4-(β-hydroxyethyl)thiazole (**25**) (15).* Sodium nitrite (1 N aqueous solution, 18.2 mL, 18.2 mmol) was added slowly to a solution of **24** (1.92 g, 12.1 mmol) in concentrated HCl (48 mL) at −10 °C. After the mixture had been stirred at room temperature for 10 min, 57 mL of water and 27 mL of 35% H_3PO_2 were added and the reaction mixture was stored at −20 °C overnight. The reaction mixture was then warmed to −5 °C, titrated to pH 10 with 10 M NaOH, and extracted with ether. The organic phase was dried (sodium sulfate), and the ether was removed in vacuo. The product was purified by flash chromatography (silica, 95:5 methylene chloride/methanol mixture) to give **25** as a yellow oil (665 mg, 4.6 mmol, 38% yield): ^{1}H NMR δ 8.596 (s, 1H), 3.966 (t, 2H), 3.3 (br, 1H), 2.91 (t, 2H), 2.415 (s, 3H).

*5-Methyl-4-(β-hydroxyethyl)thiazole Dimethyl Phosphate (**26**).* Tellurium(IV) chloride (*16*) (267 mg, 1.0 mmol) was carefully added to a mixture of thiazole **25** (178 mg, 1.2 mmol), triethylamine (172 mg, 1.7 mmol), and trimethyl phosphite (185 mg, 1.5 mmol) in 4 mL of dichloromethane. After being stirred at room temperature under argon for 1 h, the reaction mixture was filtered. The filtrate was diluted with water and extracted several times with dichloromethane. The solvent was removed in vacuo, and the product was purified twice by flash chromatography (silica, 95:5 ethyl acetate/methanol mixture) to yield **26** (191 mg, 0.76 mmol, 61% yield): ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.57 (s, 1H), 4.37 (q, 2H), 3.74 (s, 3H), 3.69 (s, 3H), 3.11 (t, 2H), 2.44 (s, 3H).

*5-Methyl-4-(β-hydroxyethyl)thiazole Phosphate Diammonium Salt (**27**).* TMS-Br (*16*) (303 μL, 352 mg, 2.3 mmol) was added dropwise to a solution of thiazole **26** (191 mg, 0.76 mmol) in dichloromethane (9 mL). The reaction mixture was stirred at room temperature for 1 h; the reaction was quenched by the dropwise addition of saturated aqueous ammonium bicarbonate (4.5 mL), and the mixture was stirred for an additional 2 h. The solvent was removed on a rotary evaporator, and the remaining residue was lyophilized until a constant weight was achieved, yielding **27** as a white solid (195 mg, 0.76 mmol, 99%): TLC (silica, 7:1:1 2-propanol/water/ammonium hydroxide); ^{1}H NMR (200 MHz, D_2O) δ 8.71 (s, 1H), 4.05 (q, 2H), 3.01 (t, 2H), 2.4 (s, 3H).

Thiamin Phosphate Synthase Assay. Thiamin phosphate synthase activity was measured using a modified version of the thiochrome assay (*3*). The reaction mixture contained 50 μM **2** (*17*), 50 μM **1**, and 2 μg of thiamin phosphate synthase in 500 μL of assay buffer [6 mM $MgSO_4$ and 50 mM Tris-HCl (pH 7.5)]. The reaction was initiated by the addition of the enzyme. Samples (60 μL) were removed at 30 s intervals; the reactions were quenched by adding 60 μL of 10% trichloroacetic acid, and the mixtures were centrifuged at 16000*g* for 2 min to remove denatured protein. The sample was then buffered by adding 100 μL of

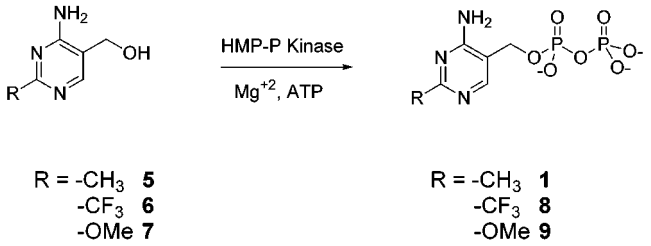


FIGURE 3: Enzymatic synthesis of the pyrimidine pyrophosphates.

supernatant to 200 μL of 4 M potassium acetate and oxidized by addition of 100 μL of freshly prepared oxidizing reagent (3.8 mM potassium ferricyanide in 7 M NaOH). The mixture was vigorously mixed and the reaction quenched after 30 s by adding 100 μL of freshly prepared 0.06% H_2O_2 in saturated KH_2PO_4. The sample was then diluted with 500 μL of doubly distilled water, and the fluorescence was measured using a Perkin-Elmer MPF-44B fluorescence spectrophotometer (excitation at 365 nm and emission at 444 nm).

Assays using **9** were carried out in an identical manner except that the excitation was carried out at 369.5 nm and the emission was measured at 439 nm.

The fluorescence for 2′-(trifluoromethyl)thiochrome **29** was quenched under these conditions. The procedure was therefore modified in the following way. Immediately before oxidation, the sample was treated with type IV-S potato acid phosphatase (100 μL of a 2 mg/mL solution) for 1 h at 37 °C, oxidized as described above, and diluted with 400 μL of doubly distilled water. The reaction mixture was extracted with 800 μL of isobutanol, and the fluorescence of the organic phase was determined (excitation at 389 nm and emission at 434 nm).

A standard thiochrome calibration curve was measured using a concentration range of 0−3 μM thiamin or thiamin analogue (**14** or **16**).

Positional Isotope Exchange (PIX) Procedure. The reaction mixture containing [^{18}O]HMP-PP, **19** (0.5 mM, prepared as described above and used immediately), and thiamin phosphate synthase (0.6 mg) in 2 mL of reaction buffer [6 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.5)] was incubated at 37 °C for either 15 min or 1 h. At the end of the incubation time, a 1 mL sample was removed and treated with bovine intestinal mucosal alkaline phosphatase [0.2 mL of a 2 mg/mL solution in 6 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.5)] at 37 °C for 1 h. The protein was removed from the sample by ultrafiltration (Centricon-10 concentrator), and the HMP was purified by HPLC on a Supelcosil SPLC-18-DB column (25 cm × 10 mm, 5 μm). Solvent A was 0.1% TFA in water, and solvent B was acetonitrile. The gradient was as follows: 98% A from 0 to 10 min and 98 to 92% A from 10 to 30 min. Under these conditions, HMP eluted at 12.8 min. The solvent was removed using a rotary evaporator at 40 °C followed by high vacuum at room temperature. The ^{18}O content of HMP was determined by electrospray ionization MS analysis by comparing the relative peak hight of the protonated parent peaks at 140 and 142 Da (solution in 50% acetonitrile in water, using a Micromass Quattro 1 triple-quadrupole tandem mass spectrometer). A control experiment, from which thiamin phosphate synthase was omitted, was carried out in an identical manner.

RESULTS

The enzymatic syntheses of **1**, its 2-trifluoromethyl analogue **8**, and its 2-methoxy analogue **9** are outlined in Figure 3. The synthesis of 2′-methoxythiamin, **14**, is outlined in Figure 4. The synthesis of 2′-(trifluoromethyl)thiamin, **16**, is outlined in Figure 5. The syntheses of [^{18}O]HMP-PP, **19**, and the isomeric thiazole **27** are outlined in Figures 6 and 7, respectively.

Thiamin phosphate synthase (*B. subtilis*) was purified from an *E. coli* overexpression strain as previously described (*3*) and assayed using the thiochrome method (Figure 8). This assay relied on the facile oxidation of thiamin to the highly fluorescent thiochrome. CF_3-thiochrome **29** and MeO-thiochrome **30** exhibited slightly different absorption and emission spectra, and the assay was therefore calibrated separately for these compounds.

Using this assay, we have determined the relative values of k_{cat} for **1** and **9**. The data are shown in Figure 9. CF_3-HMP-PP **8** was not a substrate for the enzyme even when

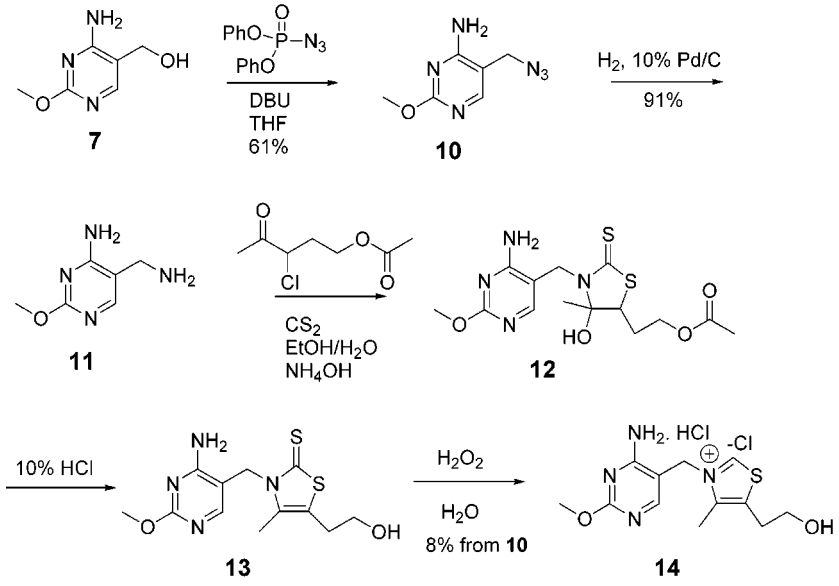


FIGURE 4: Synthesis of 2′-methoxythiamin.

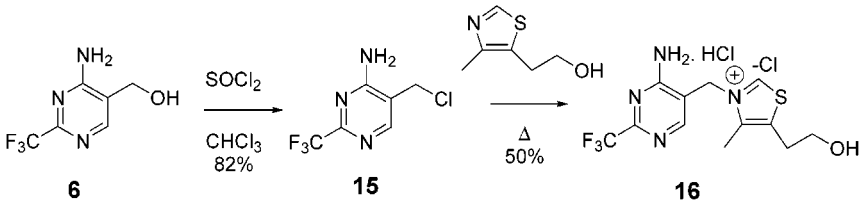


FIGURE 5: Synthesis of CF_3-thiamin.

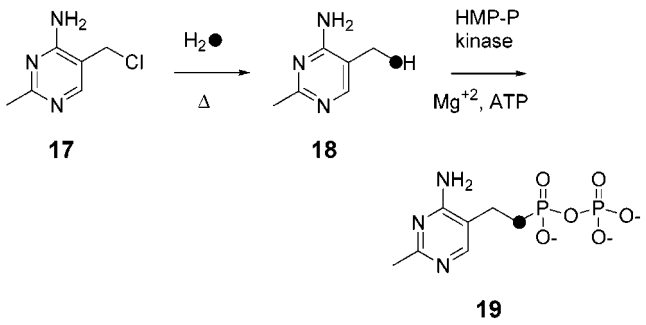


FIGURE 6: Synthesis of [^{18}O]HMP-PP.

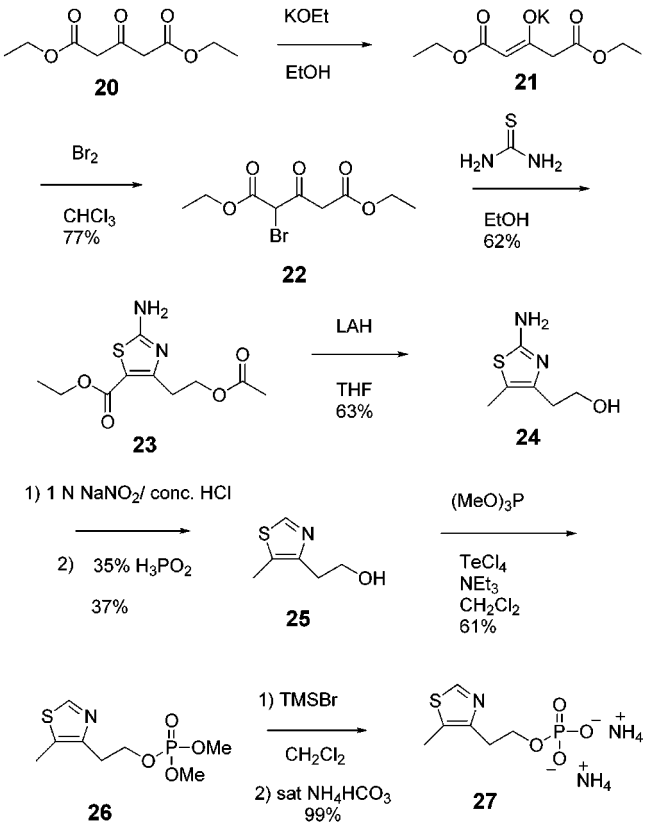
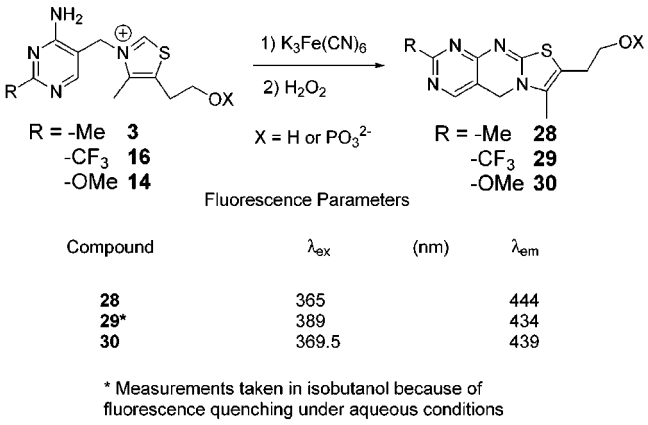


FIGURE 7: Synthesis of the isomeric thiazole phosphate.



Fluorescence Parameters

Compound	λ_{ex} (nm)	λ_{em}
28	365	444
29*	389	434
30	369.5	439

* Measurements taken in isobutanol because of fluorescence quenching under aqueous conditions

FIGURE 8: Oxidation of thiamin and thiamin analogues to the corresponding thiochromes.

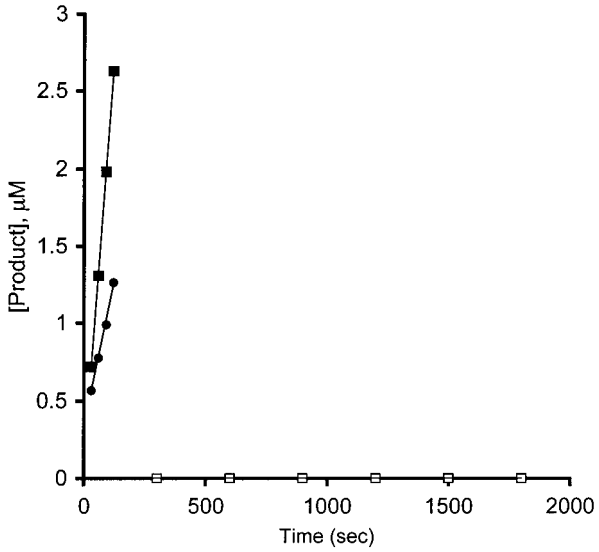


FIGURE 9: Thiamin phosphate synthase-catalyzed alkylation of Thz-P (**2**) with HMP-PP (●, **1**), MeO-HMP-PP (■, **9**), and CF_3-HMP-PP (□, **8**) under saturating conditions.

using 100 times more enzyme than the standard assay. Since the limit of sensitivity of the CF_3-thiochrome assay is 0.01 μM, this demonstrates that $k_{cat}(CH_3) > 7800\ k_{cat}(CF_3)$. The relative k_{cat} for **9** was found to be $2.8k_{cat}$ for **1**.

To test for positional isotope exchange, [^{18}O]HMP-PP **19** was synthesized as shown in Figure 6, using HMP-P kinase (*Salmonella typhimurium*). Incubation of **19** with thiamin phosphate synthase for 15 min or 1 h was followed by dephosphorylation with alkaline phosphatase, purification of HMP by HPLC, and analysis for the relative amounts of **5** and **18** by mass spectrometry (Figure 10). The MS data were corrected by subtracting the signal originating from the 49% [^{18}O]HMP-P contamination in the sample. The high level of [^{18}O]HMP-P present in the PIX reaction mixture was due to severe substrate inhibition of HMP-P kinase which occurs at levels of HMP of >0.5 mM. We needed to use these high concentrations to facilitate the detection of **5** during its purification by HPLC. Since HMP-P is not a substrate for thiamin phosphate synthase, we corrected the MS data with the assumption that this compound would not undergo an enzyme-dependent PIX. The corrected MS data for **5** and **18** originating from [^{18}O]HMP-PP were used in the analysis as given in Table 1. This corrected MS data demonstrated that 32 and 41% of the labeled oxygen of **19** was exchanged in 15 min and 1 h, respectively. A control experiment, carried out in the absence of the enzyme, demonstrated that HMP-PP was relatively stable under the conditions of the PIX experiment.

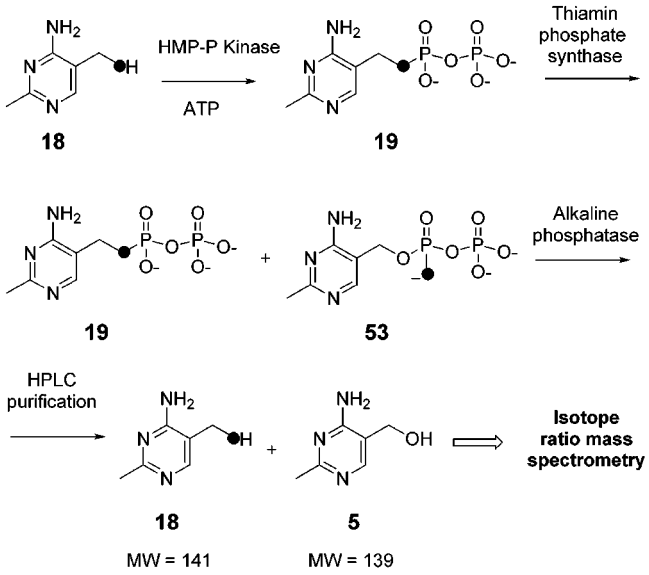


FIGURE 10: Experimental strategy used to measure the level of thiamin phosphate synthase-catalyzed positional isotope exchange of **19**.

Table 1: Corrected Mass Spectrometry Data and PIX Analysis

	15 min		60 min	
reaction mixture	% $^{16}O^a$	% PIX^b	% $^{16}O^a$	% PIX^b
no enzyme control	7.7	0	8.9	2.0
HMP-PP, iThz-P, and TPS	8.7	1.7	11.9	5.1
HMP-PP and TPS	32	41	41	54

[a] After correction for [^{18}O]HMP-P impurity. Error in all MS measurements is ±2%. [b] Percent PIX (% PIX) indicates the extent of PIX where 100% PIX corresponds to 67% ^{16}O.

DISCUSSION

The thiamin phosphate synthase-catalyzed reaction may proceed via an associative (S_N2-like) or dissociative (S_N1-like) mechanism. To differentiate between these two possibilities, we have analyzed the effect of electron-donating and electron-withdrawing substituents, at C2 of the pyrimidine, on the reaction rate (k_{cat}). If the reaction proceeds via a carbocation intermediate, k_{cat} will be very sensitive to the nature of the C2 substituent, and replacing the C2 methyl with a trifluoromethyl group or with a methoxy group should give a large rate retardation or rate acceleration, respectively. In contrast, if the reaction proceeds by an associative mechanism, these substituents will have a much smaller effect on the reaction rate. This analysis assumes that the formation of the putative carbocation is at least partially rate limiting and that the new substituents will not perturb the binding of the substrate analogues to the active site of the enzyme.

While we do not have reference data for substituent effects on S_N1 and S_N2 reactions of HMP, related substituent effects can be estimated from data in the literature (Figure 11). For example, in the S_N1 solvolysis of *tert*-cumyl chloride **31**, replacement of the *p*-methyl group with a trifluoromethyl group is predicted to retard the rate by a factor of 6250 while replacement of the methyl group with a methoxy group is predicted to accelerate the rate by a factor of 60 (*18*, *19*). The corresponding predicted relative rates for the S_N2 displacement of the chloride of *p*-(trifluoromethyl)- and

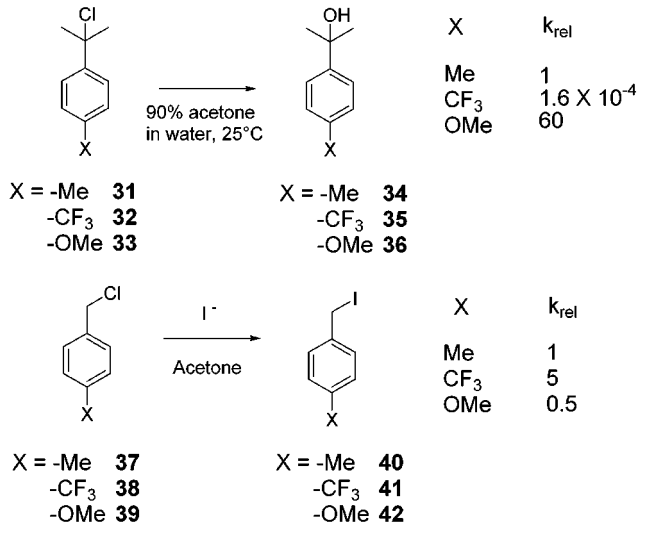


FIGURE 11: Methyl, trifluoromethyl, and methoxy substituent effects on the S_N1 and S_N2 displacement reactions of substituted benzyl chlorides.

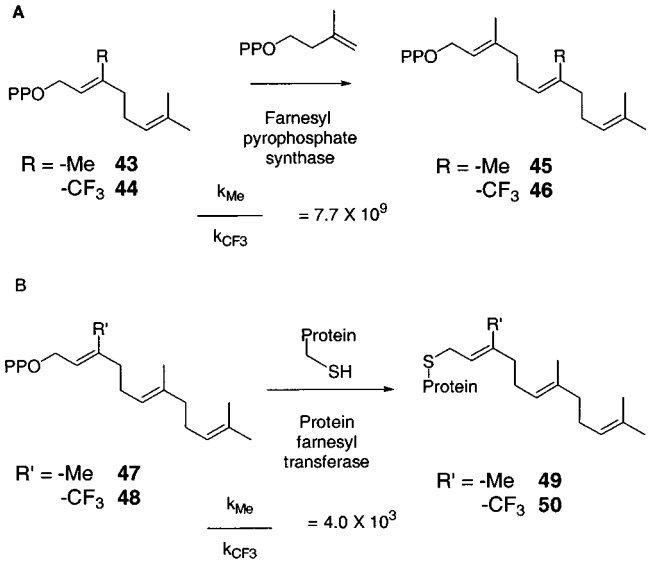


FIGURE 12: (A) Methyl and trifluoromethyl substituent effects on farnesyl pyrophosphate synthase and (B) methyl and trifluoromethyl substituent effects on protein farnesyl transferase.

p-methoxy-substituted benzyl chloride were 5 and 0.5, respectively (*19*, *20*).

An elegant example of a substituent effect in enzymology is the demonstration that the farnesyl pyrophosphate synthase-catalyzed prenyl transfer was retarded by a factor of 7.7×10^9 when one of the methyl group was replaced with a trifluoromethyl group (*21*). This suggested that this reaction proceeded via an allyl carbocation intermediate. In contrast, the corresponding rate for protein farnesyl transfersase (PFTase) is retarded by a factor of 4000, suggesting that this reaction proceeds by an associative mechanism with a "late" transition state, having partial carbocation character (*22*). The change in mechanism for the two enzymes is reasonable because PFTase uses a cysteine thiolate nucleophile which is a much stronger nucleophile than the π-electrons of a double bond (Figure 12).

For thiamin phosphate synthase, we find that k_{cat} for CF_3-HMP-PP (**8**) is at least 7800 times lower than k_{cat} for HMP-PP (**1**). This large substituent effect suggests that thiamin

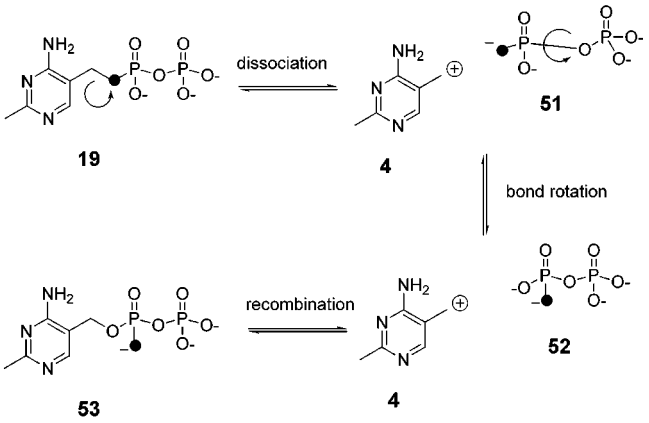


FIGURE 13: Proposed positional isotope exchange of [^{18}O]HMP-PP catalyzed by thiamin phosphate synthase.

formation occurs via a carbocation intermediate. We also found that k_{cat} for MeO-HMP-PP (**9**) was 2.8 times greater than that for HMP-PP (**1**). This small methoxy substituent effect, relative to the corresponding effect on the solvolysis of cumyl chloride, suggests either that this substituent perturbs the binding of the pyrimidine to the active site or more likely that the product dissociation has become partially rate limiting for this pyrimidine analogue.

Positional isotope exchange is also a technique widely used to probe for carbocation intermediates (*23*). In this experiment (Figure 13), formation of the ion pair of **4** and **51** from **19** could allow the pyrophosphate moiety to rotate, thus interchanging the ^{16}O and the ^{18}O. Ion recombination would generate **53** in which the ^{18}O attached to the pyrimidine has been replaced with ^{16}O. The observation of this exchange by the isolation of **19** and **53** from the enzymatic reaction mixture would provide good experimental evidence for a dissociative mechanism.

Our first attempt to measure the level of thiamin phosphate synthase-catalyzed PIX using chemically synthesized **19** (*24*) failed because this compound underwent complete PIX during purification by DEAE ion exchange chromatography. This necessitated the development of a fast enzymatic synthesis of **19**, which was accomplished using the surprising observation that HMP-P kinase also catalyzes the phosphorylation of HMP (*10*). Control experiments demonstrated only low levels of background PIX in the samples of **19** thus prepared, relative to the thiamin phosphate synthase-dependent PIX.

Our strategy for determining the level of enzyme-catalyzed PIX is outlined in Figure 10. Incubation of **19** with thiamin phosphate synthase for 15 min was followed by dephosphorylation. The relative quantities of **18** and **5** were determined by integrating the areas of the ESI-MS signals for 142 (141 + H^+) and 140 (139 + H^+). After correcting the MS data for contaminating [^{18}O]HMP-P resulting from the incomplete conversion of HMP to HMP-PP, we estimate that 32% of the ^{18}O of **19** had been replaced by ^{16}O. The extent of exchange was increased to only 41% by increasing the incubation time to 60 min (see Table 1), suggesting that the enzyme is undergoing inactivation under the reaction conditions, because 67% of the oxygen should have been scambled to ^{16}O if all of the substrate underwent PIX. In addition to the substituent effect, this result is also consistent with a dissociative mechanism.

We have also determined the level of PIX for the enzyme substrate ternary complex. To block the competing addition of the thiazole to the pyrimidine carbocation, the thiazole was replaced with an isomeric thiazole (**27**) in which the nitrogen was replaced with the less nucleophilic sulfur.

The thiazole substrate analogue **27** is a moderate inhibitor of thiamin phosphate synthase with an IC_{50} of 101 μM. When

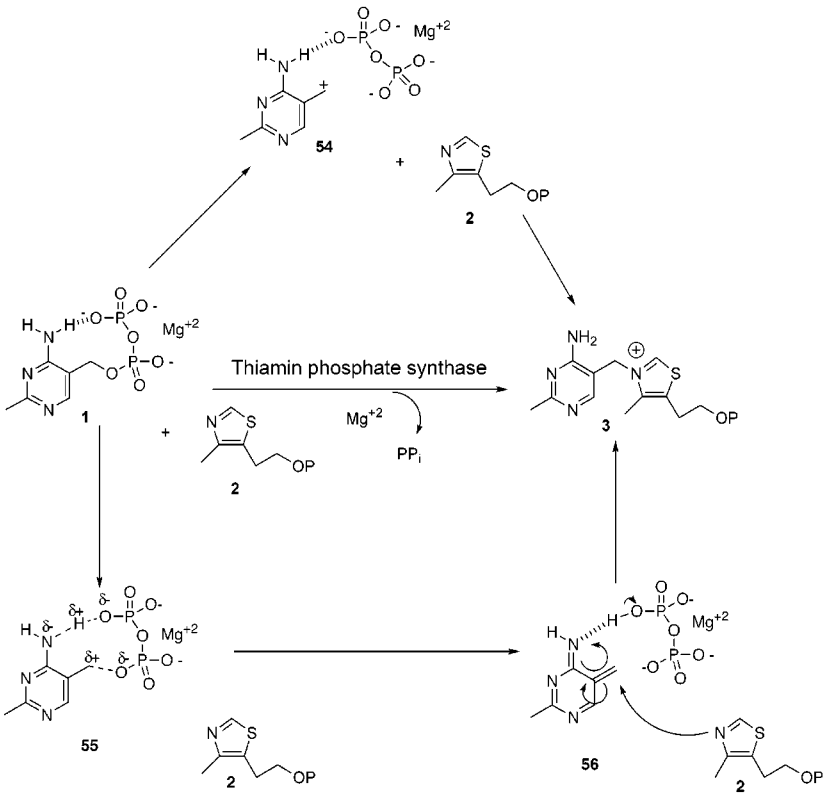


FIGURE 14: Current mechanistic proposals for thiamin phosphate synthase.

the PIX experiment was carried out in the presence of **27**, 2 and 5% PIX occurred over the course of 15 and 60 min, respectively. This experiment demonstrates that the pyrophosphate bond rotation needed to observe PIX is slower in the ternary complex than in the enzyme−HMP-PP complex.

The crystal structure of thiamin phosphate synthase complexed to thiamin phosphate and pyrophosphate demonstrates that Ser130 is hydrogen bonded to the leaving oxygen of the inorganic pyrophosphate. Removal of this hydrogen bond in the S130A mutant resulted in a >7800-fold reduction in k_{cat}. This indicates that k_{cat} is also very sensitive to the stability of the leaving group and is consistent with a dissociative mechanism.

Our current mechanistic proposal for thiamin phosphate synthase is outlined in Figure 14. The substituent effects and the large rate retardation observed for the S130A mutant demonstrate that there is considerable positive charge in the pyrimidine ring and negative charge on the oxygen leaving group in the transition state. In addition, the positional isotope exchange demonstrates the reversible cleavage of the pyrophosphate from the pyrimidine. These observations are consistent with a mechanistic proposal in which dissociation of the pyrophosphate from the pyrimidine gives the pyrimidine carbocation **54**, which is then trapped by the thiazole **2** to give thiamin phosphate **3**. The structure of the enzyme product complex suggests that the carbocation is stabilized by an electrostatic interaction with the counterion and by a hydrogen bond between the pyrimidine amino group and the pyrophosphate.

Our experimental observations are also consistent with a proposal in which proton transfer from the amino group to the pyrophosphate and C−O bond cleavage occur via an asynchronous, concerted mechanism. In the transition state for this reaction, C−O bond cleavage must occur ahead of proton transfer to account for the substituent effects. This is reasonable because the N−H bond is orthogonal to the fragmenting C−O bond. Therefore, substantial cleavage of the C−O bond must take place before the pK_a of the amine becomes sufficiently low for proton transfer to the pyrophosphate to occur. Addition of the thiazole **2** to **56**, again in a reaction involving a concerted proton transfer, would complete the reaction. The pK_a of the pyrimidine carbocation in **54** is likely to be similar to the pK_a of 4-aminopyrimidinium methiodide, which equals 12 (*25*). The fourth ionization of pyrophosphate has a pK_a of 9.1. However, the corresponding pK_a of the enzyme-bound pyrophosphate is likely to be considerably lower than this because of reduction of the negative charge on the enzyme pyrophosphate. Thus, while these pK_a values argue against the formation of **56** as an intermediate, the uncertainty in our estimates does not allow us to convincingly differentiate between **54** and **56** as intermediates.

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