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An Efficient Enzymatic Synthesis of Thiamin Pyrophosphate

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Abstract—Thiamin pyrophosphate was synthesized in 71% yield, on a multi-milligram scale, using overexpressed thiazole kinase, pyrimidine kinase, thiamin phosphate synthase, and thiamin phosphate kinase. This provides a facile route to isotopically labeled thiamin pyrophosphate from its readily available pyrimidine and thiazole precursors.

Thiamin pyrophosphate utilizing enzymes are found in all living systems. For example, in *Escherichia coli*, seven such enzymes have been identified; these play key roles in the citric acid cycle, in carbohydrate metabolism, and in the biosynthesis of isopentenyl pyrophosphate, menaquinone, pyridoxal phosphate, thiamin pyrophosphate and branched chain amino acids.[1] While the mechanistic enzymology of thiamin-utilizing enzymes has been extensively studied,[2] NMR has not been widely used as a mechanistic tool for this family of enzymes because isotopically-labeled thiamin pyrophosphate is difficult to synthesize.[3,4]

The later steps in the thiamin biosynthesis and salvage pathways are outlined in Figure 1.[5] 2-Methyl-4-amino-5-hydroxymethyl pyrimidine pyrophosphate (**3**) is formed by the iterative phosphorylation of (**2**) and 4-methyl-5-(2-hydroxyethyl)thiazole phosphate (**5**) is formed by the phosphorylation of (**4**). The pyrimidine pyrophosphate (**3**) and the thiazole phosphate (**5**) are then coupled, in a reaction catalyzed by thiamin phosphate synthase, to give (**6**). A final phosphorylation gives thiamin pyrophosphate (**1**). Alternatively, thiamin pyrophosphate can be formed by the direct pyrophosphorylation of thiamin (**7**).[6] Since all of the enzymes required for the catalysis of these reactions have now been overexpressed and characterized, and the pyrimidine[7,8] and thiazole moieties[9,10] are readily synthesized, we are able to report here the development of a facile enzymatic synthesis of thiamin pyrophosphate.

Our synthesis strategy overcomes the chemically difficult thiazole/pyrimidine coupling and the low yielding phosphorylations of thiamin to thiamin pyrophosphate.[11] The overall yield compares favorably with the less versatile synthesis of thiamin pyrophosphate from thiamin using thiamin pyrophosphokinase. This synthesis will be of use for the preparation of isotopically-labeled thiamin pyrophosphate from the readily synthesized pyrimidine and thiazole moieties for NMR studies on thiamin-utilizing enzymes.

Standard methods were used for DNA restriction endonuclease digestion, ligation and transformation of DNA.[12,13] Automated DNA sequencing was performed at the Cornell BioResource Center. Plasmid DNA was purified with the Wizard™ Plus SV DNA miniprep kit (Promega, Madison, WI, USA). DNA fragments were separated by agarose gel electrophoresis, excised and purified with the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA, USA). *E. coli* strain DH5α was used as a recipient for transformations during plasmid construction, propagation and storage. PCR was catalyzed with Platinum *Pfx* DNA polymerase and performed in a Perkin-Elmer GeneAmp PCR System 2400 (Gibco BRL, Rockville, MD, USA). All restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). *E. coli* strain BL21(DE3) and the pET overexpression system were purchased from Novagen (Madison, WI, USA).

Thiazole (**4**) and thiamin alcohol (**7**) were purchased from Sigma, pyrimidine (**2**) was synthesized as previously described.[7,8]

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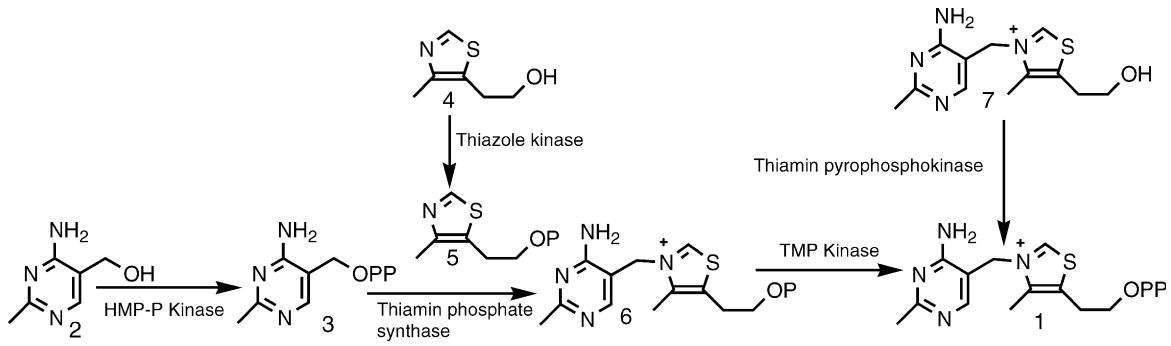

Figure 1. The later steps in the thiamin pyrophosphate biosynthesis and salvage pathways.

Construction of the Thiamin Monophosphate Kinase (*thiL*) Overexpression Plasmid

The *thiL* gene was PCR amplified from *E. coli* genomic DNA using the following primers: upstream primer 5′-GGC ATA ACA TATGGC ATG TGG CGA GTT CTC CCT G-3′ (inserts an *Nde*I site at the start codon of the *thiL* open reading frame); downstream primer 5′-CAC CAC CCT CGAGTA GAG CTG CCA GGG CAA AAA GGT-3′ (inserts an *Xho*I site after the end of the *thiL* open reading frame). The purified PCR product was digested with *Nde*I and *Xho*I, purified and ligated into similarly digested pET-16b(+) vector. Colonies were screened for the presence of the insert and a representative plasmid was designated pCLK710. The PCR-derived DNA was sequenced and shown to contain no errors.

Protein overexpressed from pCLK710 was not active, presumably due to the presence of the amino-terminal His_{10} tag. The *thiL* gene was therefore excised with *Bam*H1/*Nde*1 and transferred to the pET-22b(+) vector to give pKIS100.

Overexpression of Thiazole Kinase, HMP-P Kinase, Thiamin Phosphate Synthase and Thiamin Phosphate Kinase

Plasmids pYZK3 and pYZC6927 containing the *Bacillus subtilis* genes for thiazole kinase and thiamin phosphate synthase in pQE30 and pQE32 (Qiagen), respectively, were transformed separately into *E. coli* SG13009(pREP4).[14] Plasmids pCLK601b containing the *E. coli* gene for HMP-P kinase in pET22b(+),[15] and pKIS100, containing the *E. coli* gene for thiamin phosphate kinase in pET22b(+) were separately transformed into *E. coli* BL21(DE3). A single colony of each was grown overnight in 3 mL LB broth containing either ampicillin (200 μg/mL) for *E. coli* BL21(DE3) or ampicillin (200 μg/mL) and kanamycin (25 μg/mL) for *E. coli* SG13009. These starter cultures (0.5 mL) were used to inoculate 500 mL of LB broth containing the same concentration of antibiotics as the starter culture. Once these cultures reached an OD_{600} of 0.5, IPTG was added to a final concentration of 1 μM. All cultures were grown at 37 °C with the exception of the thiamin phosphate kinase overexpression strain which was grown at 15 °C. These induced cultures were then grown for 6 h at 37 °C with shaking and the thiazole kinase, HMP-P kinase, thiamin phosphate synthase and thiamin phosphate kinase were partially purified as described below.

Partial Purification of Thiazole Kinase, HMP-P Kinase, Thiamin Phosphate Synthase and Thiamin Phosphate Kinase

The four 500-mL cultures prepared above were combined and the cells were pelleted by centrifugation at 10,000*g* for 30 min. The cell pellet was resuspended in 40 mL of 50 mM Tris–HCl, 2 mM DTT, 2 mM EDTA, pH 8, and the cells were lysed by sonication (Heat Systems Ultrasonics model W-385 sonicator equipped with a 0.5-inch tip on a 2-s cycle, 50% duty, 3 min). Cellular debris was removed by centrifugation at 27,000*g* for 30 min, followed by passing the supernatant through a 0.45-μm filter. The cell free extract buffer was then exchanged with a volatile buffer (100 mM NH_4HCO_3, pH 8) by gel filtration on a PD-10 column (Amersham).

Overexpression and Purification of Thiamin Pyrophosphokinase

Plasmid pTPK-1, containing the *S. cerevisiae* gene for thiamin pyrophosphokinase in a pET28a(+) vector, was transformed into *E. coli* BL21(DE3).[16] A single colony of the recombinant cells was grown overnight, with shaking at 37 °C in 3 mL LB broth containing kanamycin (25 μg/mL). This starter culture (0.5 mL) was used to inoculate 500 mL LB broth containing kanamycin (25 μg/mL). Once this culture reached an OD_{600} of 0.5, IPTG was added to a final concentration of 1 μM. This induced culture was then grown for 6 h at 37 °C with shaking. The cells were pelleted by centrifugation at 10,000*g* for 30 min and the supernatant discarded. The pellet was then resuspended in 5 mL of 50 mM Tris–HCl, 2 mM DTT, 2 mM EDTA, pH 8, and the cells lysed by sonication. Cellular debris was removed by centrifugation at 27,000*g* for 30 min, followed by passing the supernatant through a 0.45 μm filter. The cell free extract buffer was then exchanged with a volatile buffer (100 mM NH_4HCO_3, pH 8) by gel filtration on a PD-10 column (Amersham).

Synthesis of Thiamin Pyrophosphate (1) from Pyrimidine (2) and Thiazole (4)

An 80 mL solution containing pyrimidine (2) (19 mg, 1.7 mM), thiazole (4) (20 mg, 1.7 mM), ATP (13.3 mM), $MgCl_2$ (25 mM), and KCl (50 mM) was prepared in 100 mM NH_4HCO_3 buffer, pH 8. The pooled cell extract (10 mL) was then added, the reaction mixture was stirred at room temperature for 12 h and the product was purified as described below.

Synthesis of Thiamin Pyrophosphate (1) from Thiamin (7)

A 40 mL solution containing thiamin alcohol (7) (21 mg, 2 mM), ATP (10 mM) and $MgCl_2$ (20 mM) was prepared in 100 mM NH_4HCO_3 buffer, pH 8. Partially purified thiamin pyrophosphokinase (5 mL) was added, the reaction mixture was stirred at room temperature for 12 h and the product was purified as described below.

Purification of Thiamin Pyrophosphate

The reaction mixture was centrifuged at 27,000*g* for 30 min, the supernatant was collected and diluted to 250 mL with 25 mM NH_4HCO_3 buffer, pH 7.6. This was then loaded at 0.33 mL/min onto 140 mL of DEAE-Sephadex A25 resin that had been washed with 300 mL of 25 mM NH_4HCO_3, pH 7.6. An 800 mL linear gradient from 25 to 600 mM NH_4HCO_3, pH 7.6, was then passed through the column at a flow rate of 2.5 mL/min, and 7.5-mL fractions were collected. Thiamin-containing fractions were identified by spotting 10 μL of each fraction onto a ceramic surface and adding 10 μL 3.9 mM $K_3Fe(CN)_6$ in 7 M NaOH to oxidize thiamin to the highly fluorescent thiochrome which was easily detected by irradiating with a long wavelength UV lamp. For the positive fractions, a 50-μL aliquot of each fraction was oxidized with 10 μL 3.9 mM $K_3Fe(CN)_6$ in 7 M NaOH and the resulting thiochromes were analyzed by HPLC to identify the phosphorylation state of the thiamin-containing fractions. The elution conditions were as follows: flow rate = 1 mL/min; 0 min, 85%A, 5%B, 10%C; 3 min, 85%A, 10%B, 5%C; 20 min, 55%A, 40%B, 5%C; 24 min, 55%A, 40%B, 5%C; 26 min, 55%A, 5%B, 40%C; 29 min, 55%A, 5%B, 40%C; 30 min, 85%A, 5%B, 10%C, A = H_2O, B = MeOH, C = 0.1 M K_3HPO_4, 4 mM Bu_4NHSO_4. Under these conditions thiochrome alcohol, thiochrome phosphate and thiochrome pyrophosphate elute after 17.7, 19.8 and 22 min, respectively. The fractions containing thiamin pyrophosphate were pooled and lyophilized. This dry powder was repeatedly suspended in 100 mL water and lyophilized to constant mass. The isolated yield of thiamin pyrophosphate from (2) and (4) was 41 mg (71%) and the yield from (7) was 26 mg (75%). The final product for both syntheses was identical to an authentic sample of thiamin pyrophosphate by HPLC, MS and NMR analysis. ^1H NMR (400 MHz, D_2O), 7.85 (s, 1H), 5.20 (s, 1H), 3.95 (q, 2H), 3.08 (t, 2H), 2.37 (s, 3H), 2.24 (s, 3H), MS ESI (m/z, M^+) 425.

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