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Overexpression, purification and characterization of two pyrimidine kinases involved in the biosynthesis of thiamin: 4-amino-5-hydroxymethyl-2-methylpyrimidine kinase and 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate kinase

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Abstract.

The overexpression, purification and characterization of 4-amino-5-hydroxymethyl-2-methylpyrimidine kinase (HMP kinase) and 4-amino-5-hydroxymethyl-2-methylpyrimidine monophosphate kinase (HMP-P kinase) are described. Surprisingly HMP-P kinase also shows HMP kinase activity. These enzymes are useful reagents for the preparation of intermediates on the thiamin biosynthetic pathway.

Keywords: Enzymes, phosphorylation, biosynthesis

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1. Introduction

The later steps in the biosynthesis of thiamin pyrophosphate (vitamin B_1) **7** are outlined in Figure 1 [1, 2, 3]. Thiamin phosphate synthase catalyzes the displacement of pyrophosphate from 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate **2** by 4-methyl-5-(β-hydroxyethyl)thiazole phosphate **4** to give thiamin phosphate **6** [4, 5, 6, 7].

Three kinases are involved in the formation of the substrates for this enzyme. Two of these, 4-amino-5-hydroxymethyl-2-methylpyrimidine kinase (HMP kinase) and 4-methyl-5-(β-hydroxyethyl)thiazole kinase (Thz kinase), are likely to be non-essential enzymes involved in the salvage of thiamin phosphate precursors from the growth medium [8,9]. The third kinase, 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate kinase (HMP-P kinase), is an essential biosynthetic enzyme [9]. A fourth kinase is required to form thiamin pyrophosphate **7**, the biochemically active form of the cofactor [10]. Thiamin kinase is also likely to be a salvage enzyme [8]. In yeast, thiamin is converted directly to thiamin pyrophosphate in a reaction catalyzed by thiamin pyrophosphokinase [11, 12].

The genes coding for PdxK [13], ThiD [9], ThiM [4, 9, 14, 15], ThiK [16], ThiL [10] and Thi80 [11, 12] have been cloned but, with the exception of ThiM and ThiL, have not yet been overexpressed. An HMP kinase (not necessarily PdxK) has been purified from wild type *Escherichia coli* and also catalyzes the phosphorylation of pyridoxine, pyridoxal and pyridoxamine [17, 18]. ThiM and Thi80 have also been purified [4, 5, 12]. ThiD, ThiK and ThiL have not yet been purified. Here we describe the overexpression, purification and characterization of HMP kinase (PdxK) and HMP-P kinase (ThiD).

Compound **2** is reactive and difficult to purify [19]. The availability of HMP kinase and HMP-P kinase, in reagent quantities, would open up a facile enzymatic route to this important intermediate for thiamin biosynthesis.

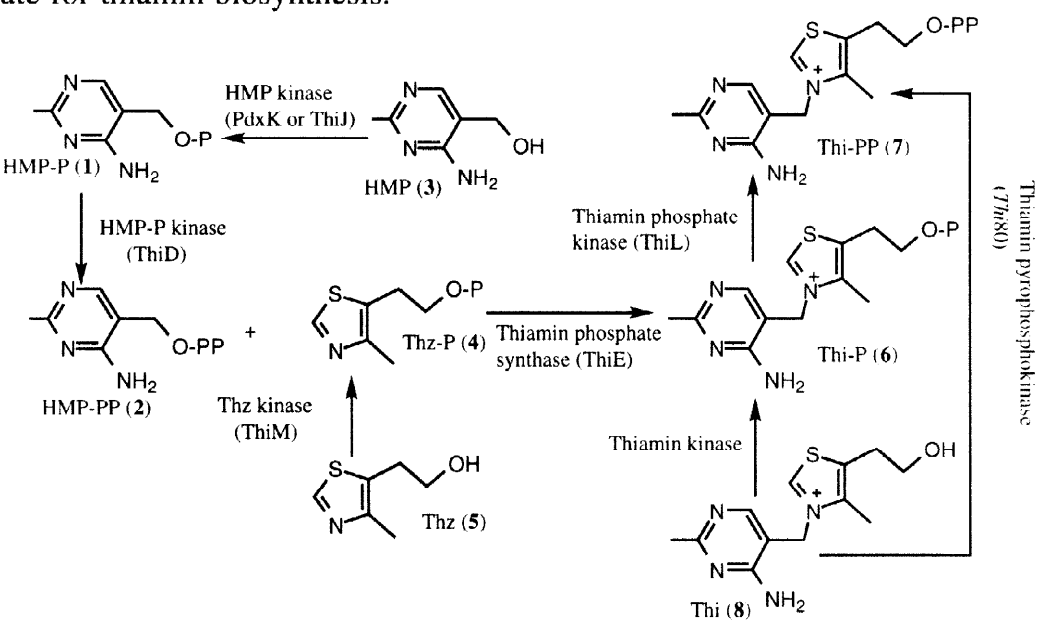

Figure 1. The later steps in the biosynthesis of thiamin pyrophosphate. (The gene product names are given in parenthesis under the enzyme name).

2. Results and discussion

HMP-P kinase and HMP kinase were both overexpressed at a high level as soluble proteins (Figure 2). The subunit mass of HMP-P kinase, as determined by SDS-PAGE analysis, is 30 kDa (31,232 Da predicted) and of HMP kinase is 33 kDa (33,534 Da predicted). Gel filtration indicates that both enzymes are tetrameric in the native state.

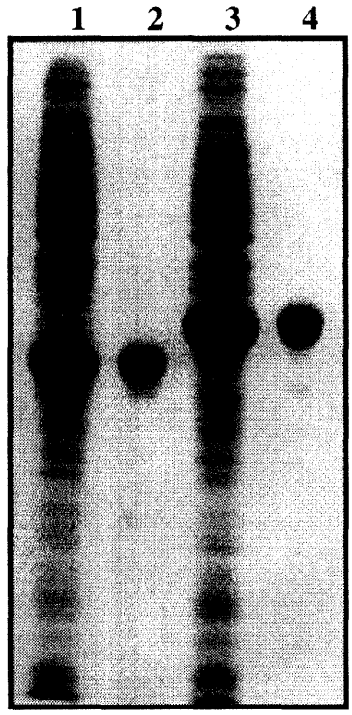

Figure 2. SDS-PAGE of HMP-P kinase and HMP kinase purification. Lane 1: HMP-P kinase crude extract. Lane 2: Ni^{+2}-NTA column purification of HMP-P kinase. Lane 3: HMP kinase crude extract. Lane 4: Ni^{+2}-NTA column purification of HMP kinase.

HPLC analysis of the reaction mixture resulting from the treatment of **3** with ATP in the presence of HMP kinase demonstrated the formation of a new product that comigrated with a synthetic sample of **1**. The identity of this product was confirmed by NMR analysis of the reaction mixture. The ^{31}P NMR spectrum showed the expected singlet due to **1** at 5.15 ppm and the ^{1}H-NMR spectrum showed a doublet at 4.42 ppm for the methylene protons of **1**. The methyl and vinylic proton resonances were masked by other components in the reaction mixture. This analysis demonstrates that HMP kinase (PdxK) catalyzes the phosphorylation of **3**. This enzyme is probably different from the previously isolated HMP kinase [17] because the subunit molecular masses of the two enzymes are significantly different (33kDa for PdxK vs. 43kDa). The kinetic parameters for this reaction as well as for the phosphorylation of pyridoxine are shown in Figure 3A. A representative Lineweaver Burk plot is shown in Figure 3B.

A)

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat} / K_m (s^{-1} μM^{-1})
3	33.5	.43	.013
pyridoxine	28.5	.36	.0126
ATP	143.1[a]	.43[a]	.003[a]

[a]Parameters were measured using 3 as other substrate.

B)

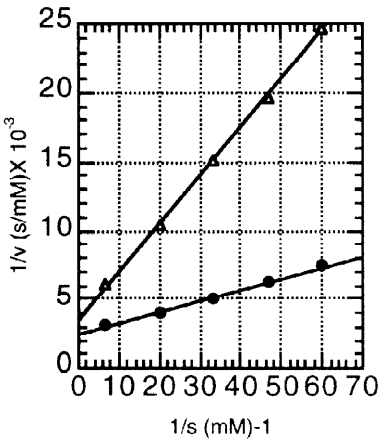

Figure 3. A) Kinetic parameters for HMP kinase. B) Typical Lineweaver-Burk plots for HMP kinase. •, pyrimidine as variable substrate; Δ, ATP as variable substrate.

Chromatographic analysis of the reaction mixture resulting from the treatment of **1** with ATP in the presence of HMP-P kinase demonstrated the formation of a new product that comigrated with a synthetic sample of **2**. The identity of this product was confirmed by NMR analysis of the reaction mixture. The ^{31}P NMR spectrum showed the two expected doublets at -5.0 ppm and -9.2 ppm and the ^{1}H-NMR spectrum showed a doublet at 4.86 ppm for the methylene protons of **2**. The methyl and vinylic proton resonances were masked by other components in the reaction mixture. This analysis demonstrates that ThiD catalyzes the phosphorylation of **1** and confirms at the biochemical level the results of the genetic analysis [9]. The kinetic parameters for this reaction are shown in Figure 4A and a representative Lineweaver-Burk plot is shown in Figure 4B.

Incubation of HMP-P kinase with **3** and ATP shows formation of **1** and **2** by HPLC. In addition, the ADP assay demonstrates that **3** is a substrate for HMP-P kinase and that complete phosphorylation results in the consumption of 2 mol of ATP per mole of **3**. The kinetic parameters for this reaction are shown in Figure 4A. This second kinase activity is unprecedented for kinases and is surprising because the active sites required for the two phosphorylation reactions are expected to be quite different. This observation and the fact that both ThiD and ThiJ have been mapped to the same region of the *E.coli* chromosome strongly suggest that ThiD may also carry out the function previously assigned to ThiJ [20].

The high level overexpression and facile purification of HMP kinase and HMP-P kinase described here will greatly facilitate the synthesis of derivatives of **2** for use as mechanistic probes of thiamin phosphate synthase.

A)

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
3	150	.086	.001
1	23.3	.070	.006
ATP	193.0[a]	.070[a]	.0007[a]

[a]Parameters were measured using 1 as other substrate.

B)

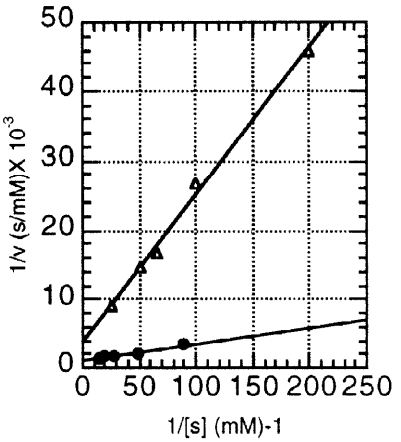

Figure 4. A) Kinetic parameters for HMP-P kinase. B) Typical Lineweaver-Burk plots for HMP-P kinase. •; pyrimidine as variable substrate, Δ; ATP as variable substrate.

3. Experimental

Materials.

E. coli strain BL21(DE3), the pET overexpression system and HisBind protein purification kit were purchased from Novagen. Luria-Bertani medium was purchased from Gibco BRL Products. Gel filtration resin was purchased from Bio-Rad as 100-200 mesh agarose beads, having an operating range of 10,000 to 1,500,000 Da. Centricon concentrators and ultrafiltration membranes were purchased from Amicon. Pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, NADH, pyridoxine and gel filtration standards were purchased from Sigma. Buffer A is 20 mM Tris-HCl, pH=8. Buffer B is 20 mM Tris-HCl, pH=7.5. Buffer C is triethanolamine HCl, pH=8.

4-Amino-5-hydroxymethyl-2-methylpyrimidine phosphate, 1 and 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate, 2. To a suspension of **3** [21, 22] (200 mg, 1.44 mmol) in dry acetonitrile (3 mL) was first added bis(triethylammonium) hydrogen phosphate (580 mg, 2.03 mmol) followed by trichloroacetonitrile (760 μL, 7.6 mmol) in two portions separated by 20 minutes. The mixture was allowed to stir overnight at room temperature. Water (5 mL) was added to the resulting bright yellow solution and the mixture was washed with ethyl acetate (5 x 5 mL). The aqueous layer was dried *in vacuo* at room temperature. Aqueous ammonium bicarbonate (25 mM, pH=8, 3 mL) was added and this solution applied to an anion exchange DEAE A-25 column (1.5 x 28 cm) that had been equilibrated with aqueous ammonium bicarbonate (25 mM, pH=8, 300 mL). A linear concentration gradient of ammonium bicarbonate buffer (25 mM to 600 mM, over 200 mL,

flow rate = 2 mL/min) was applied to the column while 8 mL fractions were collected. Unreacted **3** eluted first, followed by **1** and lastly **2**. UV active fractions were analyzed by silica gel TLC (7:1:1 isopropanol:water:ammonium hydroxide) R_f (**3**)=0.8, R_f (**1**)=0.2 and R_f (**2**)<0.1. The appropriate fractions were pooled and repeatedly lyophilized until a constant weight was achieved. **1** was obtained as 172 mg (0.774 mmol) of the diammonium salt and **2** was obtained as 74 mg (0.211 mol) of the triammonium salt for a combined yield of 68%. ^{1}H-NMR (D_2O) of **1**: δ 8.0 (s, 1H), 4.42 (d, 2H) and 2.46 (s, 3H). ^{31}P-NMR (H_3PO_4 external standard) of **1**: δ 5.15 (s, 1P). ^{1}H-NMR (D_2O) of **2**: δ 8.1 (s, 1H), 4.86 (d, 2H) and 2.5 (s, 3H). ^{31}P-NMR (H_3PO_4 external standard) of **2**: δ -5.0 (d, 1P) and –9.2 (d, 1P).

Overexpression of pdxK *and* thiD.

For the construction of the PdxK overexpression plasmid, the *pdxK* gene from pTX485 [13] was PCR amplified (upstream mutagenesis primer: 5'-GAA AAA ATC ATA TGA GTA GTT TGT TGT TGT TTA ACG-3'; downstream mutagenesis primer: 5'-CCC TGA CTC GAG TTG TTA TCT CCA ACA TGG ACG-3', the introduced *Nde*I and *Xho*I sites are underlined). The amplified fragment was purified by agarose gel electrophoresis and cloned into the *Nde*I and *Xho*I sites of pET-16b(+) to give pCLK701.

For the construction of the ThiD overexpression plasmid, the *NdeI/BamHI* fragment from the PCR reaction with pThiD-5 [9] as the template (upstream mutagenesis primer: 5'- GGA GGC GGC ATA TGC AAC GAA TTA ACG CGC TGA CG-3'; downstream mutagenesis primer: 5'- TAG GAT CCA TAC TAC CAC CAC GCG TGG AAA TGA TGT AC-3', the introduced *Nde*I and *Xho*I sites are underlined) was cloned into the *Nde*I and *BamHI* sites of pET-16b(+) to give pCLK601. The entire insert for pCLK701 and pCLK601 was sequenced to verify that no mutations had occurred during the PCR process.

For overexpression, pCLK701 and pCLK601 were transformed into competent *E. coli* BL21(DE3). The transformed cells were grown in LB medium containing 200 mg/L of ampicillin at 37°C until the OD_{595} reached 0.6. At this time, the culture was induced by the addition of isopropylthiogalactoside (IPTG) to a final concentration of 1 mM and the temperature was reduced to 25°C. After overnight growth with shaking, the cells were harvested by centrifugation at 7500*g* for 30 min. and the cell pellets were stored at -70°C.

Purification of HMP kinase and HMP-P kinase.

Expression of pCLK601 and pCLK701 yields proteins with a His-tag fused to the N-terminus. For the purification of HMP kinase, the cell pellet, derived from 250 mL of induced culture, was lysed and purified according to the Novagen protocol for the purification of His-tagged proteins [23]. The protein was eluted directly from the HisBind column into 100 mL buffer B to prevent precipitation. This solution was dialyzed overnight against 4 L of buffer B, concentrated to 10 mg/mL by ultrafiltration (YM 10 membrane) and stored in 10% glycerol at -70°C. The protein lost half of its activity after one month of storage. The yield of HMP kinase from 250 mL of culture was 17 mg.

The purification of HMP-P kinase was carried out in an identical manner except that dilution into buffer B was not required. The enzyme was stored as a 10 mg/mL solution in buffer A containing 10% glycerol at -70°C. This protein also lost half of its activity after one month of storage. The yield of HMP-P kinase from 1 L of culture was 80 mg. The SDS-PAGE gel of the overexpression and purification of both kinases is shown in Figure 2.

Native molecular mass determination by gel filtration.

The native molecular mass of both proteins was determined by gel filtration, using Bio-Gel agarose beads with a 10,000 to 1,500,000 Da operating range. Buffer A was used as the elution buffer. Elution volumes were normalized using blue dextran to determine the void volume. The corrected elution volumes were used to determine the mass of the protein using a standard curve derived from proteins of known molecular mass. The standards used were carbonic anhydrase (29,000 Da), bovine serum albumin (66,000 Da), alcohol dehydrogenase (150,000 Da) and α-amylase (200,000 Da).

Identification of the HMP kinase and HMP-P kinase reaction products by ^{1}H- and ^{31}P-NMR.

The complete reaction mixture (in 2 mL), contained 0.7 mM ATP, 0.7 mM **3** or **1**, 1.4 mM $MgCl_2$, 1.4 mM KCl in buffer C. Enzyme was added to a final concentration of 1.2 μM. The reaction mixture was incubated at 25°C for 3 hours, filtered through a Centricon-10 concentrator to remove protein and lyophilized overnight. The sample was then dissolved in 650 μL D_2O and transferred to an NMR tube. The NMR spectra were measured using a 400 MHz Varian NMR Spectrometer. The ^{31}P-NMR spectra were proton decoupled and H_3PO_4 was used as an external standard. H_2O was the reference for the ^{1}H spectra. Analysis of the HMP-P kinase reaction mixture required water suppression.

Identification of the HMP kinase and HMP-P kinase reaction products by HPLC.

HMP kinase and HMP-P kinase reactions were run as described in the previous section and quenched by the addition of 100 μL of acetone. The solution was centrifuged to remove protein. Twenty μL of this mixture were injected onto a Supelcosil LC-18-T HPLC column and eluted under isocratic conditions with 0.1 mM sodium phosphate buffer, pH = 6 at a flow rate of 1 mL/min. The reaction products were identified by comparison with synthetic samples of **1** (retention time = 2.75 min) and **2** (retention time = 2.30 min).

Kinetic analysis of HMP kinase and HMP-P kinase.

Both kinases produce ADP as one of the reaction products. This can be assayed by monitoring NADH consumption using a pyruvate kinase/lactate dehydrogenase coupled enzyme system (Figure 5) [24].

For the determination of the apparent K_m and V_{max} of **3**, pyridoxine and **1** in the presence of saturating concentrations of ATP, the assay mixture consisted of 5 mM ATP, 16.7-150 mM

of **3**, pyridoxine or **1**, 10 mM $MgCl_2$, 50 mM KCl, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 7 units/mL pyruvate kinase and 10 units/mL lactate dehydrogenase in 0.5 mL. For the determination of the apparent K_m and V_{max} of ATP in the presence of saturation concentrations of **3**, pyridoxine or **1**, the assay mixture consisted of 16.7-150 mM ATP, 0.2 mM of **3**, pyridoxine or **1**, 10 mM $MgCl_2$, 50 mM KCl, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 7 units/mL pyruvate kinase and 10 units/mL lactate dehydrogenase in 0.5 mL. **3**, pyridoxine and **1** showed substrate inhibition with an IC_{50} of approximately 1 mM. ATP showed no inhibition at 5 mM.

Each of these components was prepared as a concentrated stock solution in buffer C. The reaction was initiated at 25°C by the addition of HMP kinase to a final concentration of 0.95 μM or HMP-P kinase to a final concentration of 12.5 μM. The ADP-dependent consumption of NADH was monitored continuously at 340 nm and initial velocities were determined by measuring the initial slope of the time-course. Control experiments demonstrated that pyruvate kinase and lactate dehydrogenase were not limiting in any of the assays carried out. Reactions assayed in the absence of pyrimidine substrates showed negligible ATPase activity for both enzymes. The data were analyzed with KaleidaGraph, *v* 3.0.1.

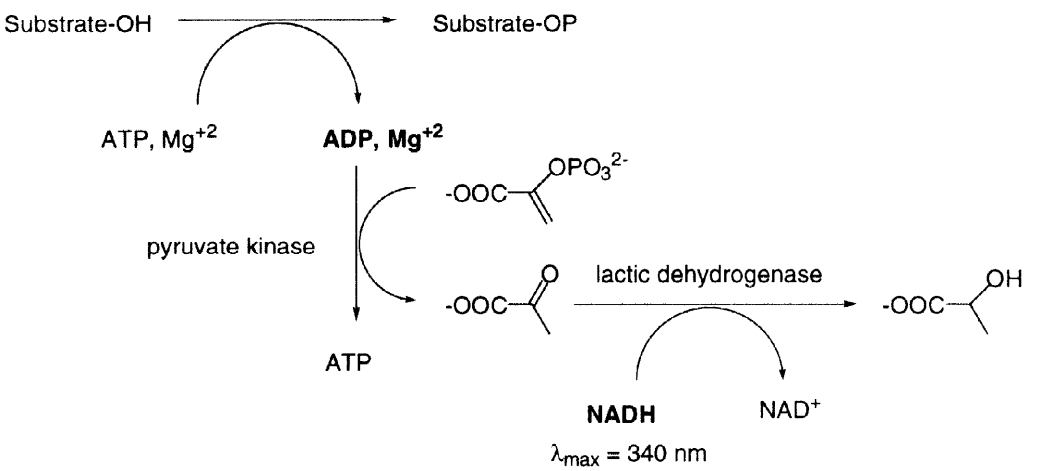

Figure 5. Coupled enzyme assay for HMP kinase and HMP-P kinase.

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