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Characterization of Two Kinases Involved in Thiamine Pyrophosphate and Pyridoxal Phosphate Biosynthesis in *Bacillus subtilis*: 4-Amino-5-Hydroxymethyl-2-Methylpyrimidine Kinase and Pyridoxal Kinase

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Two *Bacillus subtilis* genes encoding two proteins (currently annotated ThiD and YjbV) were overexpressed and characterized. YjbV has 4-amino-5-hydroxymethyl-2-methylpyrimidine and 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate kinase activity and should be reannotated ThiD, and *B. subtilis* ThiD has pyridoxine, pyridoxal, and pyridoxamine kinase activity and should be reannotated PdxK.

The biosynthesis of thiamine pyrophosphate (TPP) involves the coupling of 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (HMP-PP) and 4-methyl-5-β-hydroxyethylthiazole phosphate (Thz-P) to form thiamine phosphate followed by a final phosphorylation (1). In addition to the de novo biosynthesis, microorganisms have developed several salvage pathways for the biosynthesis of TPP (Table 1). Thiamine from the growth medium is either phosphorylated by thiamine kinase or pyrophosphorylated by thiamine pyrophosphokinase (J. Melnick, E. Lis, J.-H. Park, H. Mori, C. Kinsland, J. Perkins, G. Schyns, A. Osterman, and T. P. Begley, submitted for publication). The pyrimidine and thiazole components can also be salvaged: thiazole is phosphorylated by thiazole kinase (2, 4, 6), HMP is phosphorylated to HMP-P by both ThiD and PdxK (3, 7, 10), and the phosphorylation of HMP-P is catalyzed by ThiD (5, 6, 7). Thus, ThiD has both a biosynthetic and a salvage function in thiamine biosynthesis. PdxK is able to phosphorylate a broad range of substrates, including HMP, pyridoxal (PL), pyridoxamine (PM), and pyridoxine (PN), and is a salvage enzyme in the biosynthesis of thiamine as well as that of PL phosphate (PLP).

A search of the *Bacillus subtilis* genomic database (http://genolist.pasteur.fr/SubtiList/index.html) shows homologues of *Escherichia coli* ThiD and PdxK named YjbV (1246149–1246961) and ThiD (3899983–3900795). They are both 271-amino-acid proteins. *yjbV* is located immediately downstream of the *thiOSGF* operon that is involved in Thz-P biosynthesis, while *thiD* is not clustered with any of the thiamine or PLP biosynthetic genes. *E. coli* PdxK shows 24 and 25% sequence identity with *B. subtilis* YjbV and ThiD, respectively, and *E. coli* ThiD shows 41 and 35% identity with *B. subtilis* YjbV and ThiD, respectively. The level of sequence homology between these two proteins is too high to allow the preferred substrate to be predicted for either protein. However, the occurrence of *yjbV* in the thiazole biosynthetic operon suggests that these proteins are incorrectly annotated and that YjbV might function as the *B. subtilis* HMP/HMP-P kinase. Here we report the overexpression of YjbV and ThiD from *B. subtilis* and the identification of the substrate preferences of the two proteins.

The amino acid sequences of *E. coli* ThiD and PdxK were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and used with the SubtiList World Wide Web server for a BLAST search. For cloning *B. subtilis thiD* and *yjbV*, standard DNA restriction endonuclease digestion, ligation, and transformation methods were used (9). Genomic DNA and plasmid DNA were purified with a Wizard Plus SV genomic DNA kit and a DNA Miniprep kit, respectively (Promega). DNA fragments were separated by agarose gel electrophoresis, excised, and purified with a QIA-

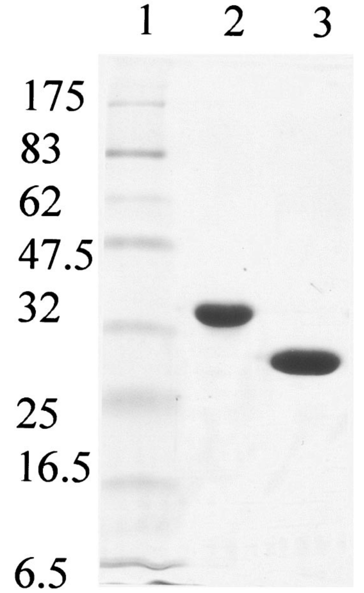

FIG. 1. SDS-PAGE (12%) analysis of purified *B. subtilis* ThiD and YjbV. Lane 1, molecular mass markers (in kilodaltons); lane 2, His-tagged ThiD; lane 3, His-tagged YjbV. Although ThiD and YjbV are predicted to have the same molecular mass, they migrate differently on the gel.

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TABLE 1. Microbial thiamine salvage enzymes

Enzyme	Microorganism	Substrate	Product	Reference(s)
ThiM	*Bacillus subtilis* *Escherichia coli* *Salmonella typhimurium*	Thz	Thz-P	2, 4, 6
PdxK	*Escherichia coli*	HMP	HMP-P	10
ThiD	*Escherichia coli*	HMP	HMP-P	7
ThiK	*Escherichia coli*	Thiamine	Thiamine phosphate	Melnick et al., submitted
ThiN	*Bacillus subtilis*	Thiamine	TPP	Melnick et al., submitted

quick gel extraction kit (Qiagen). pET-16b plasmid was obtained from Novagen. *E. coli* strain DH5α was used as a recipient for transformation during plasmid construction and for plasmid propagation and storage. *E. coli* BL21(DE3) was purchased from Novagen and used as a host strain for the overexpression of the proteins. A Perkin Elmer GeneAmp PCR System 2400 apparatus and Platinum Pfx DNA polymerase (Gibco Life Technologies) were used for PCR. *B. subtilis* CU1065 genomic DNA was used as a template for PCR. Primer synthesis and DNA sequencing were performed by the Bioresource Center at Cornell University. Primers introduced *Nde*I and *Xho*I restriction enzyme sites at the 5′ and 3′ ends, respectively.

For the overexpression and purification of ThiD and YjbV, their corresponding overexpression plasmids were transformed into competent *E. coli* BL21(DE3) cells and the transformed cells were grown at 37°C in Luria-Bertani medium containing 50 mg of ampicillin/liter. To induce the overexpression of proteins, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture (when the optical density at 595 nm reached 0.6) to achieve a final concentration of 1 mM. Culture growth was continued for 8 h at 28°C, after which the cells were harvested and stored at −80°C until further use. The proteins were purified according to a Qiagen protocol for the purification of His-tagged proteins. The eluted proteins were rapidly desalted using a PD-10 column (Amersham Pharmacia) because of instability under high-salt concentrations and stored in 5% glycerol at −80°C. ThiD was soluble and stable in 50 mM Tris buffer (pH 8), but YjbV solutions rapidly became turbid. The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the purified proteins are shown in Fig. 1. Although the migration characteristics of the purified proteins were different, their molecular weights were confirmed by mass spectrometry (data not shown).

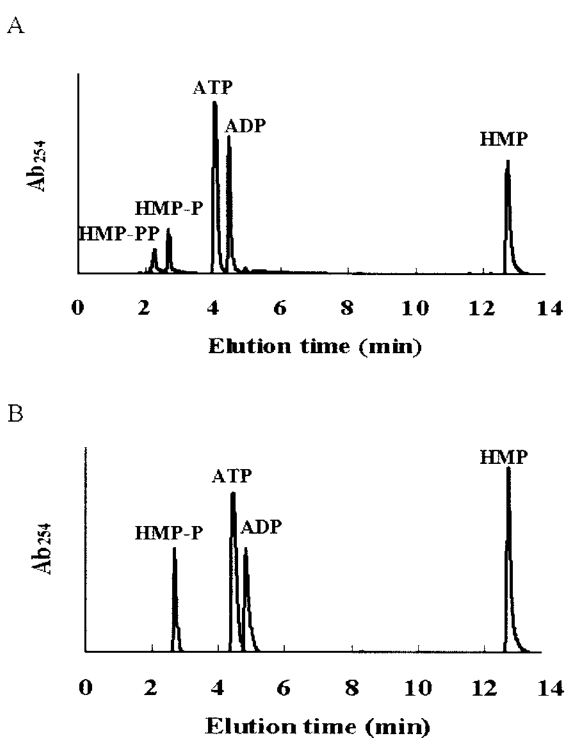

FIG. 2. HPLC analysis of the ThiD- and YjbV-catalyzed reactions. (A) YjbV catalyzed phosphorylation of HMP and HMP-P. (B) ThiD catalyzed phosphorylation of HMP.

The reaction mixtures for *B. subtilis* ThiD and YjbV enzymatic assays contained 1 mM ATP, 1 mM HMP, 2 mM $MgCl_2$, and 40 μg of enzyme in 100 μl of 50 mM Tris-HCl (pH 8). After incubation at 37°C for 10 min, the reaction was quenched by the addition of 100 μl of 10% trichloroacetic acid and centrifuged to remove proteins. A total of 20 μl of the reaction mixture was analyzed by high-pressure liquid chromatography (HPLC) (Supelcosil LC-18-T) (15- by 4.6-mm column). The elution conditions were as follows: flow rate, 1 ml/min; elution time, 0 to 20 min; elution buffer, 100% of 0.1 M potassium phosphate (pH 6.6). To conduct a competition assay, ThiD was incubated with all four substrates (0.3 mM concentrations each of HMP, PL, PM, and PN) for 30 min under the conditions described above (except that 2 mM ATP was used and the reaction mixture was analyzed by HPLC).

For kinetic studies, ADP produced by the kinase activity of ThiD or YjbV was assayed using a pyruvate kinase-lactate dehydrogenase-coupled system (which uses ADP and NADH as substrates). The consumption of NADH by this coupled system can be measured by monitoring the decrease in absorbance at 340 nm (7). Pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, NADH, and PL were purchased from Sigma. HMP was synthesized as previously described (8). The assay mixture for the kinetic analysis of ThiD in the presence of HMP or PL contained saturating concentrations of ATP (5 mM), 30 to 400 μM HMP (or 30 to 300 μM PL), 10 mM $MgCl_2$, 50 mM KCl, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 8 units of pyruvate kinase/ml, and 10 units of lactate dehydrogenase/ml in 0.6 ml of 50 mM Tris-HCl (pH 8). Addition of ThiD to achieve a final concentration of 6.7 μM initiated the reactions, which were then monitored over 5 min for NADH consumption at 340 nm.

HPLC analysis of the reaction mixture containing *B. subtilis*

TABLE 2. Kinetic parameters for substrate phosphorylation by *B. subtilis* ThiD

Substrate	K_m (μM)	k_{cat} (s-1)	k_{cat}/K_m (s-1 μM-1)
HMP	2,030	0.36	1.8×10^{-4}
PL	46.6	0.032	6.9×10^{-4}

YjbV showed the appearance of two new peaks corresponding to HMP-P and HMP-PP (Fig. 2A). The reaction mixture containing *B. subtilis* ThiD showed only one pyrimidine product peak, which corresponds to HMP-P (Fig. 2B). In addition to the phosphorylation of HMP, *B. subtilis* ThiD was able to phosphorylate PL, PM, and PN, producing PLP, PMP, and PNP, respectively. Under similar conditions, YjbV did not catalyze the phosphorylation of these compounds (data not shown). A competition assay using the substrates of ThiD revealed a preference for PL followed by HMP, PN, and PM (8:2.4:1.1:1 product ratios). The kinetic parameters for *B. subtilis* ThiD are shown in Table 2. The kinetic parameters of *B. subtilis* YjbV could not be determined, because the reaction mixture became turbid immediately after the reaction began.

Overall our results indicate that *B. subtilis* YjbV has HMP/HMP-P kinase activity and should be reannotated ThiD (i.e., the name should be changed from YjbV to ThiD) and that *B. subtilis* ThiD has PN/PL/PM/HMP kinase activity and should be reannotated PdxK (i.e., from ThiD to PdxK).

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