The PduX Enzyme of Salmonella enterica Is an L-Threonine Kinase Used for Coenzyme B\textsubscript{12} Synthesis*  

Chenguang Fan and Thomas A. Bobik

From the Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa 50011

Here, the PduX enzyme of Salmonella enterica is shown to be an L-threonine kinase used for the de novo synthesis of coenzyme B\textsubscript{12} and the assimilation of cobyric acid (Cby). PduX with a C-terminal His tag (PduX-His\textsubscript{6}) was produced at high levels in Escherichia coli, purified by nickel affinity chromatography, and partially characterized. \textsuperscript{31}P NMR spectroscopy established that Escherichia coli carries out the de novo synthesis of coenzyme B\textsubscript{12} and the assimilation of cobyric acid (Cby). PduX with a C-terminal His tag (PduX-His\textsubscript{6}) was produced at high levels in Escherichia coli, purified by nickel affinity chromatography, and partially characterized. \textsuperscript{31}P NMR spectroscopy established that

The B\textsubscript{12} coenzymes (adenosylcobalamin, AdoCbl, and methylcobalamin, MeCbl) are required cofactors for at least 15 different enzymes that are widely distributed in nature and are essential for human health (1, 2). AdoCbl and MeCbl are synthesized de novo by certain prokaryotes and from corrinoid precursors by a broader range of organisms (1, 2). The synthesis of B\textsubscript{12} has been conducted in any system.

In S. enterica, AdoCbl and MeCbl are required cofactors for three enzymes (5). MeCbl-dependent methionine synthase is used to convert homocysteine to methionine (14); AdoCbl-dependent diol dehydratase and ethanolamine ammonia lyase are required for growth on 1,2-propanediol (1,2-PD) and ethanolamine, respectively (15, 16). The genes specific for 1,2-PD utilization (pdu) are found in a large contiguous cluster (15, 17). Bioinformatic studies tentatively suggest that the last gene of the pdu operon (pduX) encodes an L-Thr kinase (18). The PduX protein has homology to the GHMP (galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase) family of kinases and a number of PduX homologues are encoded by genes located proximal to genes for AdoCbl biosynthesis. However, no experimental studies of PduX homologues have been conducted in any system.

Here, we present genetic and biochemical studies that show PduX is an L-Thr kinase used for the de novo synthesis of AdoCbl and the assimilation of Cby. To our knowledge, PduX is the first enzyme shown to phosphorylate free L-threonine and the first L-threonine kinase shown to function in coenzyme B\textsubscript{12} synthesis.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Antibiotics, Cbi, L-Thr-P, (R)-1-amino-2-propanol (AP), nucleoside triphosphates, and nucleoside diphosphates were purchased from Sigma. Isopropyl β-D-thiogalactopyranoside was from Diagnostic Chemical Ltd., Charelottetown, Prince Edward Island, Canada. Restriction enzymes and T4 DNA ligase were from New England Biolabs.

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\textsuperscript{1} To whom correspondence should be addressed. Tel.: 515-294-4165; Fax: 515-294-0453; E-mail: bobik@iastate.edu.

\textsuperscript{2} The abbreviations used are: Cbi, cobinamide; Cby, cobyric acid; AP-P, (R)-1-amino-2-propanol-O-2-phosphate; 1,2-PD, 1,2-propanediol; HPLC, high pressure liquid chromatography; AP, (R)-1-amino-2-propanol.
Bacterial Strains used in this study are listed in Table 1. The minimal medium used was NCE supplemented with 0.4% 1,2-PD, 1 mM MgSO₄, 50 µM ferric citrate, 1 µM 5,6-dimethylbenzimidazole, and 3 mM each valine, isoleucine, leucine, and threonine. LB (Luria-Bertani) medium was the rich medium used.

General Molecular Methods—Agarose gel electrophoresis was performed as described previously (19). Plasmid DNA was purified by Qiagen (Chatsworth, CA) products according to the manufacturer’s instructions. Following restriction digestion and PCR amplification, DNA was purified by Promega Wizard PCR Preps (Madison, WI). Restriction digests were carried out using standard protocols (19). For ligation of DNA fragments, purified by Qiagen (Chatsworth, CA) products according to the manufacturer’s instructions. Following gel electrophoresis, Coomassie Brilliant Blue R-250 was used to stain proteins. The protein concentration of solutions was determined by using Bio-Rad protein assay reagent.

Protein Methods—PAGE was performed by using Bio-Rad Ready gels and Bio-Rad Mini-Protean II electrophoresis cells according to the manufacturer’s instructions. Following gel electrophoresis, Coomassie Brilliant Blue R-250 was used to stain proteins. The protein concentration of solutions was determined by using Bio-Rad protein assay reagent.

Construction of Plasmids for Production of PduX and PduX-His₆—PCR was used to amplify the pduX coding sequence from pEM55 (17). The primers used for amplification were 5’-GGCGCAGATCTATGCCGCACACTTCCG-TACCCT-3’ and 5’-GGCGCAGAATCTTACGTGACGTT-TGACCGCGCCA-3’. The reverse primer used for fusing six histidine residues to the C terminus of PduX was 5’-GGCGGC-CAAGCTTATCAATGATGATGATGATGTGACTGCA- TTGACCGCGCA-3’. These PCR primers introduced BglII and HindIII restriction sites that were used for cloning into vector pTA925 or pLaC22 (20). Following ligation, clones were introduced into Escherichia coli DH5α by electroporation, and transformants were selected by plating on LB agar supplemented with 25 µg ml⁻¹ kanamycin (pTA925) or 100 µg ml⁻¹ ampicillin (pLaC22). Pure cultures were prepared from selected transformants. The presence of insert DNA was verified by restriction analysis or PCR, and the DNA sequence of selected pduX clones was determined. Clones with the expected DNA sequence were used for further study.

DNA Sequencing—DNA sequencing was carried out by the DNA facility of Iowa State University Office of Biotechnology using automated sequencing equipment from Applied Biosystems Inc.

Growth Curves—Growth media are described in the figure legends. To prepare the inoculum, 2 ml LB cultures were incubated overnight at 37 °C, and then cells were collected by centrifugation and suspended in growth curve medium. Media were inoculated to a density of 0.15 absorbance units, and cell growth was monitored by measuring the optical density at 600 nm using a BioTek Synergy microplate reader and 48-well flat-bottom plates (Falcon). Each well was inoculated with 0.3 ml of culture and incubated at 37 °C as described (21).

Corrinoid Extraction and Quantification—Selected strains of S. enterica were grown aerobically on 5 ml of LB medium supplemented with 1% 1,2-PD, 1 µM 5,6-dimethylbenzimidazole, and 5 µM CoCl₂ overnight at 37 °C in 17 × 100-mm test tubes. Cells were collected by centrifugation and suspended in 1 ml of 50 mM Tris, pH 7.0. The cell suspension was transferred into 3-ml serum vials. The vials were sealed and flushed with helium for 3 min. The suspension was autoclaved for 10 min at 121 °C, then placed on ice for 10 min and centrifuged at 25,000 × g for 1 h at 4 °C. The corrinoids present in the supernatant were quantified using a bioassay based on the AdoCbl-dependent growth of S. enterica strain BE86 on the ethanolamine minimal medium. Growth measurements were carried out using a microplate reader as described above for “growth curves.” Quantitation was based on a standard curve of vitamin B₁₂ concentration versus maximum cell density of BE86. The assay was linear from 0 to 20 nm vitamin B₁₂ with an r² value of 0.9843.

Growth of pduX Expression Strains—The E. coli strains used for expression of pduX were grown on 400 ml of LB supplemented with 25 µg ml⁻¹ kanamycin at 16 °C in a New Brunswick Scientific, Innova 4230, refrigerated shaker incubator with shaking speed at 275 rpm. Cells were grown to an absorbance of 0.6—0.8 at 600 nm, and protein expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside. Cells were incubated at 16 °C with shaking at 275 rpm for an additional 18 h and harvested by centrifugation at 5000 × g for 10 min at 4 °C using a Beckman JA-10 rotor.

### TABLE 1

| Species and strain | Genotype | Source |
|--------------------|----------|--------|
| E. coli DH5α       | F⁻λ⁻endA1hsdR17relA1supE44 thi-1 recA1 gyrA96 relA1 ΔlacZYA-argF169 (d800lacZΔM15) | This study |
| BL21DE3 RIL       | F⁻ompT hsdS (rK− mK−) dcm− Ter− galA (DE3) endA Hfe [argII leuW CamR'] | T.A. Bobik lab collection |
| BE119              | BL21DE3 RIL/pTA925 | Stratagene |
| BE945              | DH5α/pTA925-pduX-His₆ | This study |
| BE963              | BL21DE3 RIL/pTA925-pduX-His₆ | This study |
| S. enterica serovar Typhimurium LT2 | | |
| BE86               | DEL1715[::pLac22] | J.R. Roth lab collection |
| BE200              | Del pduX688 | This study |
| BE287              | pLac22 | T.A. Bobik lab collection |
| BE438              | cbiB2x-mudI | This study |
| BE892              | Del pduX685:Kan | This study |
| BE933              | Del pduX685:Kan/pLac22 | This study |
| BE935              | Del pduX685:Kan/pLac22-pduX | This study |
| BE938              | Del pduX686 cobD498::mudI | This study |

Beverly, MA. Pefabloc SC PLUS was purchased from ICN Biomedicals, Inc., Aurora, OH. Other chemicals were from Fisher Scientific. Cby was kindly provided by Kathy Krasny from the laboratory of J. Escalante-Semerena.
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Preparation of Cell Extract of S. enterica—About 2.0 g of cell paste was suspended in 8 ml of 50 mM Tris, pH 7.5, 300 mM NaCl, 20 mM imidazole and broken by French press (Thermo Electron Corp.) at 20,000 psi. Pefabloc SC PLUS was added to the cell extract to a concentration of 100 μg ml⁻¹ to inhibit proteases. The crude cell extract was centrifuged at 35,000 × g for 30 min at 4 °C using a Beckman JA-17 rotor to separate the soluble and insoluble fractions. The supernatant was the soluble fraction used for enzyme purification.

Purification of pduX-His₆—8 ml of soluble cell extract from an E. coli expression strain was loaded onto a column containing 5 ml of nickel-nitrioltriacetic acid Superflow resin (Qiagen) previously equilibrated with 50 mM Tris, pH 7.5, 300 mM NaCl, 20 mM imidazole. The column was washed with 5 ml of 50 mM Tris, pH 7.5, 300 mM NaCl, 300 mM imidazole. The purified protein was desalted by using PD-10 desalting column (GE Healthcare Life Sciences) and eluted with 3.5 ml of 10 mM Tris, pH 7.5, 300 mM NaCl, 300 mM imidazole. The purified protein was reconstituted with 1 mg of purified PduX-His₆ in a final volume of 1 ml.

Enzyme Assay—The activity of PduX was measured by an ADP Quest assay kit according to the manufacturer’s instructions (DiscoverRx, Fremont, CA). The assay uses a coupled enzyme reaction system to generate hydrogen peroxide from ADP (ADP reacts with PEP to form pyruvate by pyruvate kinase, and then pyruvate oxidase generates hydrogen peroxide). Hydrogen peroxide when combined with acetyl dihydroxy phenoxazine in the presence of peroxidase generates the fluorescently active resorufin dye. Standard assay mixtures contained 15 mM HEPES, pH 7.4, 20 mM NaCl, 10 mM MgCl₂ in a total volume of 0.1 ml. Activity was determined by monitoring the fluorescence intensity with a BioTek Synergy HT microplate reader. The excitation wavelength was 530 nm and emission wavelength was 590 nm. To minimize background fluorescence, 96-well black microplates were used (Greiner Bio-one, Frickenhausen, Germany). For kinetic studies, the concentrations of certain assay components varied as indicated in the text. Quantitation was based on standard curves made with ATP, GTP, CTP, or UTP.

³¹P NMR Spectroscopy—The products of the PduX reaction were analyzed by ³¹P nuclear magnetic resonance (NMR) spectroscopy. Reactions were performed using conditions described for the PduX enzyme assay with 1 mg of purified PduX-His₆ in a final volume of 1 ml. The reaction mixtures were incubated at 37 °C. Protein was removed by a Vivaspin 500, 10K filtration device. Samples were transferred to 5-mm NMR tubes (WILMAD). 50 μl of 100% D₂O was added (finally 5%). ³¹P NMR spectra of reference compounds and kinase reaction products were obtained with a 11.7 tesla magnet (Biomolecular Nuclear Magnetic Resonance Facility, Iowa State University, Ames, IA) and a Bruker DRX 500 spectrometer at the following settings: frequency, 202.347 MHz; excitation pulse width, 8.1 μs; pulse repetition delay, 6 s; and spectral width, 12.136 kHz. Spectra were processed with TOPSPIN 1.3 software from Bruker. Chemical shifts were referenced to triphenyl phosphate, which was set to −18.0 ppm.

RESULTS

PduX Is Needed for S. enterica Growth on 1,2-PD Minimal Medium Supplemented with Cby, but Not on Similar Medium Supplemented with Cbi—L-Thr kinase is predicted to be required for the synthesis of AdoCbl from Cby, but not from Cbi (Fig. 1). Therefore, we measured aerobic growth of S. enterica and a pduX deletion mutant (BE892) on 1,2-PD minimal medium supplemented with Cby or Cbi. Under these conditions, growth requires the synthesis of AdoCbl from Cby or Cbi (15).

Results showed that a pduX mutant was significantly impaired for growth on 1,2-PD minimal medium supplemented with Cby, but grew well on similar medium supplemented with Cbi (Fig. 2). In contrast, wild-type S. enterica grew well on 1,2-PD medium supplemented with either Cby or Cbi. The generation times of S. enterica with Cby and Cbi were 4.2 and 5.8 h, whereas those of the pduX mutant were 4.2 and 17.8 h, respectively. These are the expected phenotypes of an L-Thr kinase mutant. Normal growth with Cbi indicates that...
the 1,2-PD degradative pathway is intact, and that corrinoid uptake is unimpaired because a single system is used for uptake of Cbi, Cby, and other corrinoids (6–8). It also shows that pduX mutants can carry out all the steps needed for the conversion of Cbi to AdoCbl (Fig. 1). Thus, the inability of pduX mutants to use Cby can be attributed to a defect in the conversion of Cby to AdoCbi-phosphate (Fig. 1). This process requires four enzymes: 1) an adenosyltransferase (CobA); 2) an enzyme that converts AdoCby to AdoCbi-phosphate (CbiB); 3) an l-Thr-P decarboxylase (CobD); and 4) an l-Thr kinase (9, 13, 22). The CobA, CbiB, and CobD enzymes have been characterized in S. enterica (9, 13, 22). Hence, the above studies suggest that PduX is an l-Thr kinase used for the conversion of Cby to AdoCbi.

A pduX Deletion Mutation Is Complemented by Ectopic Expression of pduX—A pduX expression plasmid (pLac22- pduX) was introduced into a pduX deletion mutant by electroporation. This plasmid fully corrected the growth defect of a pduX mutant on 1,2-PD minimal medium supplemented with Cby (the generation time was 6.9 h compared with 6.1 h for wild-type) (Fig. 3). In contrast, plasmid without insert had little effect on growth (the generation time is 17.4 h). This showed that the observed phenotype (poor growth with Cby) resulted from the pduX mutation but not from polarity or an unknown mutation.

Supplementation of Growth Media with l-Threonine Phosphate Substantially Corrects the Growth Defect of a pduX Mutant—In many cases, biosynthetic mutants are corrected by supplementation of growth medium with a downstream metabolite. Studies conducted here showed that 100 μM l-Thr-P substantially corrected the growth defect of a pduX mutant on 1,2-PD minimum media containing Cby (Fig. 4). With 100 μM l-Thr-P the generation time for a pduX deletion mutant was 8.8 h compared with a generation time of 8.3 h for the wild-type strain. Both strains reached a maximum optical density at 600 nm of about 0.4. These results indicate that PduX has a role in the synthesis of l-Thr-P.

**AP Is Ineffective for Correction of a pduX Deletion Mutant**—Prior studies showed that a cobD mutant was partly corrected for the synthesis of MeCbl from Cby by addition of AP to growth media (23). This and subsequent findings indicated that an unknown kinase phosphorylated AP to AP-P bypassing the cobD defect (Fig. 1) (13). Therefore, PduX was tested for AP kinase activity. However, results were negative. Both a cobD mutant and a pduX cobD double mutant grew similarly on minimal glucose medium supplemented with Cby and AP indicating that S. enterica converts AP to AP-P in the absence of PduX (data not shown). Concentrations of AP between 1 and 20 mM were tested and in no case did the PduX+ strain grow better than the PduX– strain. The studies described above were conducted in a metE background under conditions where the conversion of Cby to MeCbl is required for growth in minimal medium (24). Tests of PduX for AP kinase activity using 1,2-PD minimal medium supplemented with Cby and enzyme assays with purified recombinant enzyme were also negative (data not shown). Thus, results indicate that PduX does not phosphorylate AP.

**PduX Is Needed for S. enterica Growth on Minimal Ethanolamine Medium Supplemented with Cby, but Not on Similar Medium Supplemented with Cbi**—The pduX gene is the last gene in the pdu operon (17). Measurable expression of this operon requires 1,2-PD (15, 25–27). This raised the question of...
whether PduX supports ethanolamine degradation. Results showed that a pduX mutant was unable to grow on ethanolamine minimal medium supplemented with Cby, but grew well on similar medium supplemented with Cbi (Fig. 5). In contrast, wild-type S. enterica grew well on ethanolamine medium supplemented with either Cby or Cbi. The generation times of S. enterica with Cbi and Cby were 4.3 and 4.4 h, whereas those of the pduX mutant were 4.3 and 33.1 h, respectively. In addition, the growth defect of the pduX mutant on ethanolamine with Cby was fully corrected by ectopic expression of PduX, showing that PduX supports ethanolamine degradation. Results also showed that a pduX mutant was unable to grow on ethanolamine minimal medium supplemented with Cby, but grew well on similar medium supplemented with Cbi (Fig. 5). In contrast, wild-type S. enterica grew well on ethanolamine medium supplemented with either Cby or Cbi. The generation times of S. enterica with Cbi and Cby were 4.3 and 4.4 h, whereas those of the pduX mutant were 4.3 and 33.1 h, respectively. In addition, the growth defect of the pduX mutant on ethanolamine with Cby was fully corrected by ectopic expression of PduX, this defect resulted from the pduX mutation, but not from polarity or an unknown mutation (data not shown). Hence, pduX is used for AdoCbl synthesis even in the absence of 1,2-PD, which is required for transcription of the pdu operon (15, 17, 26, 27). This finding is surprising to us because it suggests that pduX has a different regulatory pattern than upstream genes presumed to be in the same operon.

**Thr-P but Not AP Supplementation Allows Growth of a pduX Mutant on Ethanolamine with Cby—**The growth defect of a pduX mutant on minimal ethanolamine medium supplemented with Cby was corrected by addition of Thr-P, but not by addition of AP-P (data not shown). This was similar to results obtained with 1,2-PD (above) and provided further evidence that PduX is an \( \gamma \)-Thr kinase.

**Production of MeCbl Sufficient to Support Methionine Biosynthesis Is Independent of pduX—**S. enterica pduX mutants require MeCbl for methionine biosynthesis; hence, \( \gamma \)-Thr kinase should be required for growth of metE strains on minimal medium supplemented with Cby (Fig. 1) (14). However, results showed that a metE pduX double mutant grew only slightly slower than wild-type on minimal glucose medium supplemented with Cby. Doubling times for the wild-type, metE mutant, and metE pduX double mutant were 2.0, 2.0, and 3.8 h, respectively. This suggested that PduX contributed to MeCbl synthesis, but S. enterica also produced a second \( \gamma \)-Thr kinase that allowed substantial growth of a metE mutant as is further explained under “Discussion.”

**PduX Is Used for the de Novo Synthesis of B12—**The triplet corresponding to the phosphate group of \( \gamma \)-Thr-P (Fig. 7) shows the intermediate reaction mixture after 30 min of incubation at 37 °C with 200 \( \mu \)g of purified PduX-His\(_8\) enzyme. The triplet centered at 11.2 ppm corresponds to the phosphate of ATP; the two doublets centered at 22.5 and 7.2 ppm correspond to the \( \gamma \)- and \( \alpha \)-phosphates of ATP, respectively. Fig. 7D shows the complete reaction mixture after 2 h of incubation at 37 °C with 200 \( \mu \)g of purified PduX-His\(_8\) enzyme. The triplet corresponding to the \( \beta \)-phosphate of ATP (−22.5 ppm) is absent. The two doublets centered at −7.2 and −11.2 ppm of the complete reaction mixture correlate well with the two doublets of the ADP standard (Fig. 7B). The singlet at 2.3 ppm corresponds to the phosphate group of \( \gamma \)-Thr-P (Fig. 7C). Fig. 7F shows the intermediate reaction mixture after 30 min of incubation at 37 °C with 200 \( \mu \)g of purified PduX-His\(_8\). It shows synthesis of AdoCbl and MeCbl by S. enterica, a pduX mutant and a cbiB mutant (as negative control). These strains produced B12, in the following amounts (picomole/g of wet cells): 2620 ± 112, 280 ± 11, and undetectable, respectively. Thus, a pduX mutant produced about 11% as much B12 as the wild-type strain. S. enterica BE86 was used for the bioassay. This strain grows on ethanolamine minimal medium supplemented with complete corrinoids such as AdoCbl and MeCbl, but not on similar medium supplemented with corrinoids such as AdoCby-P, which might accumulate in a pduX mutant.

**High-Level Expression of the PduX-His\(_8\) Protein—**PduX with a C-terminal His\(_8\) tag (PduX-His\(_8\)) was produced at high levels via an E. coli T7 expression system and purified by nickel-affinity chromatography (Fig. 6). This procedure allowed the isolation of PduX-His\(_8\) that appeared nearly homogeneous following Coomassie staining. The apparent molecular mass of PduX-His\(_8\) by SDS-PAGE was ~35 kDa, which was close to the expected value of 33.7 kDa.

**ADP and \( \gamma \)-Thr- \( \gamma \)-O-3-phosphate Are the Products of the PduX-His\(_8\) Reaction—**\(^{31}\)P NMR spectroscopy was used to identify the products of the PduX reaction. Fig. 7A shows the \(^{31}\)P NMR spectrum of a kinase reaction mixture prior to addition of purified PduX-His\(_8\) enzyme. The triplet centered at −22.5 ppm corresponds to the \( \beta \)-phosphate of ATP; the two doublets centered at −7.1 and −11.6 ppm correspond to the \( \gamma \)- and \( \alpha \)-phosphates of ATP, respectively. Fig. 7B shows the \(^{31}\)P NMR spectrum of the ADP standard. The two doublets centered at −7.2 and −11.2 ppm correspond to the \( \beta \)- and \( \alpha \)-phosphates of ADP, respectively. Fig. 7D shows the complete reaction mixture after 2 h of incubation at 37 °C with 200 \( \mu \)g of purified PduX-His\(_8\). The triplet corresponding to the \( \beta \)-phosphate of ATP (−22.5 ppm) is absent. The two doublets centered at −7.2 and −11.2 ppm of the complete reaction mixture correlate well with the two doublets of the ADP standard (Fig. 7B). The singlet at 2.3 ppm corresponds to the phosphate group of \( \gamma \)-Thr-P (Fig. 7C). Fig. 7F shows the intermediate reaction mixture after 30 min of incubation at 37 °C with 200 \( \mu \)g of purified PduX-His\(_8\). It shows
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enzyme activity was also determined. Results showed that the rate of reaction was proportional to PduX-His6 concentration from 0.006 to 6 μM when 200 μM ATP and 500 μM L-Thr were used as substrates. Linear regression yielded an $r^2$ value of 0.998. $K_m$ and $V_{max}$ Values for PduX—

Purified PduX-His6 displayed Michaelis-Menten kinetics with respect to both ATP and L-Thr (Fig. 8). Based on nonlinear regression, the $K_m$ values for ATP and L-Thr were 54.7 ± 5.7 and 146.1 ± 8.4 μM, respectively. The enzyme $V_{max}$ was 62.8 ± 3.6 nmol min$^{-1}$ mg of protein$^{-1}$ when L-Thr was varied and 61.4 ± 3.6 nmol min$^{-1}$ mg of protein$^{-1}$ when ATP was varied. The average of these two values is 62.1 ± 3.6 nmol min$^{-1}$ mg of protein$^{-1}$. Similar kinetic constants were derived from double-reciprocal plots. The $K_m$ values for ATP and L-Thr were 60.9 ± 9.7 and 127.0 ± 11.3 μM, respectively. $V_{max}$ values were 58.6 ± 6.2 nmol min$^{-1}$ mg of protein$^{-1}$ when L-Thr was varied and 57.2 ± 5.8 nmol min$^{-1}$ mg of protein$^{-1}$ when ATP was varied. The average of these two values is 57.9 ± 6.0 nmol min$^{-1}$ mg of protein$^{-1}$. When the $K_m$ values for ATP were determined, saturating levels of L-Thr (500 μM) were added to assay mixtures while varying the concentration of ATP. Similarly, saturating levels of ATP (200 μM) were added to assays when the $K_m$ values for L-Thr were determined. Purified PduX-His6 was used at a concentration of 3 μM. The values used for kinetic calculations were the average of three measurements of the initial reaction rate.

DISCUSSION

Work done in a number of laboratories has defined many steps in the de novo synthesis of AdoCbl and MeCbl (3, 5, 28–32). Of particular relevance to this work are the studies reported by Brushaber et al. (13), which showed the CobD enzyme catalyzes the decarboxylation L-Thr-P to AP-P. This finding predicted that $B_{12}$ synthesis would require an L-Thr kinase. Subsequently, Rodionov et al. (18) proposed that PduX homologues might be L-Thr kinases involved in $B_{12}$ synthesis. This was based on sequence similarity to the GHMP family of kinases and the observation that $pduX$ genes were proximal to $B_{12}$ biosynthetic genes in several instances. However, it is well known that functional predictions based on sequence similarity and gene proximity are tentative. In addition, a number of PduX homologues are encoded by genes unlinked to $B_{12}$ biosynthetic
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NMR spectroscopy indicated that purified PduX-His6 catalyzes ATP pool sizes in rapidly growing cells (1–3 mM) (33). The determined for other B12 biosynthetic enzymes that have relatively low activity in accord with the low levels of AdoCbl and MeCbl that are required to support B12-dependent processes. Sequence similarity indicates that PduX is the first enzyme reported to transfer a phosphoryl group to free l-Thr. This helps explain the paradoxical observation that S. enterica synthesizes B12 only in the absence of oxygen, but requires oxygen for degradation of ethanolamine and 1,2-PD as sole carbon and energy sources (5).

The genetic tests performed here also indicated that a pduX mutant synthesized enough MeCbl to support substantial growth of a metE mutant. This suggests that S. enterica expresses an l-Thr kinase in addition to PduX, but raises the question why is this second kinase unable to support ethanolamine or 1,2-PD degradation (Figs. 2 and 5). One explanation is that the second kinase is induced during growth on glucose. Alternatively, the activity of the second kinase might be insufficient for 1,2-PD and ethanolamine degradation. A pduX mutant grew slowly on 1,2-PD minimal medium supplemented with Cby (Fig. 2) suggesting a second kinase with low activity. Prior studies showed that the MeCbl needed to support growth of a metE mutant (methionine biosynthesis) was about 100-fold less than the amount of AdoCbl needed to support 1,2-PD and ethanolamine degradation. Hence, a second l-Thr kinase that has low activity compared with PduX is indicated.

To our knowledge, PduX is the first enzyme reported to transfer a phosphoryl group to free l-Thr. Sequence similarity indicates that PduX is a member of the GHMP kinase family (18). This family includes members that transfer the γ-phosphoryl group of ATP to acceptors such as mevalonate, homoserine, galactose, and developmental protein Xol-1 (36). A feature of PduX we think is interesting is that it phosphorylates free l-Thr, which is also required for protein synthesis. Presumably, PduX would need to be regulated to prevent l-Thr depletion or a futile cycle that needlessly consumes ATP. However, regulation of the activity of the PduX enzyme has not been studied.

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FIGURE 8. Kinetic analyses of PduX. Enzyme assays were performed with 3 μM purified PduX-His6 protein. When the Vmax value for ATP was determined, l-threonine was held at 500 μM and the concentration of ATP was varied. When the Km value for l-threonine was determined ATP was held at 200 μM and the concentration of l-threonine was varied. The values shown are the average of three measurements of the initial reaction rate. The insets are the double-reciprocal plots of the kinetic data.
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