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Purification and Initial Characterization of the *Salmonella enterica* PduO ATP:Cob(I)alamin Adenosyltransferase

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The PduO enzyme of *Salmonella enterica* is an ATP:cob(I)alamin adenosyltransferase that catalyzes the final step in the conversion of vitamin B_{12} to coenzyme B_{12}. The primary physiological role of this enzyme is to support coenzyme B_{12}-dependent 1,2-propanediol degradation, and bioinformatic analysis has indicated that it has two domains. Here the PduO adenosyltransferase was produced in *Escherichia coli*, solubilized from inclusion bodies, purified to apparent homogeneity, and partially characterized biochemically. The K_m values of PduO for ATP and cob(I)alamin were 19.8 and 4.5 μM, respectively, and the enzyme V_{max} was 243 nmol min^{-1} mg of $protein^{-1}$. Further investigations showed that PduO was active with ATP and partially active with deoxy-ATP, but lacked measurable activity with other nucleotides. ^{31}P nuclear magnetic resonance established that triphosphate was a product of the PduO reaction, and kinetic studies indicated a ternary complex mechanism. A series of truncated versions of the PduO protein were produced in *Escherichia coli*, partially purified, and used to show that adenosyltransferase activity is associated with the N-terminal domain. The N-terminal domain was purified to near homogeneity and shown to have biochemical properties and kinetic constants similar to those of the full-length enzyme. This indicated that the C-terminal domain was not directly involved in catalysis or substrate binding and may have another role.

Coenzyme B_{12} (adenosyl-cobalamin [AdoCbl]) and closely related corrinoid compounds have a broad but uneven distribution in nature (1, 20, 24). These coenzymes are used by higher animals and many bacteria, but are apparently absent from plants and fungi. The primary physiological role of AdoCbl is in the catabolism of small molecules, including certain amino acids and carbohydrates Many prokaryotes synthesize this coenzyme de novo; however, higher animals and numerous other microbes depend on the assimilation of exogenous complex precursors such as vitamin B_{12} (CNCbl).

The proposed pathways for CNCbl assimilation are thought to be similar in prokaryotes and eukaryotes (Fig. 1). The pathway initiates with the hydrolytic removal of the cyano-group by β-ligand transferase to form HOCbl or glutathionylcobalamin. β-Ligand transferase activity has been detected in rat liver, rabbit spleen, human leukocytes, human fibroblasts, and *Propionibacterium shermanii* and *Clostridium tetanomorphum* cell extracts (4, 18, 33); however, this enzyme or its encoding gene has not been isolated from any system (18). Assimilation proceeds with two one-electron reductions of the cobalt atom of HOCbl (which is in the 3^+ oxidation state) to form cob(II)alamin and then cob(I)alamin. The reductases that catalyze these reactions have been identified in a variety of systems by biochemical assay, but genetic evidence supporting their roles in AdoCbl synthesis has not been reported (9, 13, 21, 28, 30–32). The final step in CNCbl assimilation is catalyzed by an ATP:cob(I)alamin adenosyltransferase which transfers a 5′-deoxyadenosyl moiety from ATP to the cobalt atom of cob(I)alamin, to form AdoCbl (5, 7, 10, 13, 25, 27). In addition to its role in the assimilation of CNCbl, this pathway is also needed for recycling of HOCbl formed from the breakdown of AdoCbl, which is unstable in vivo (12).

Bioinformatic analyses have identified three families of adenosyltransferases that are unrelated in amino acid sequence: EutT-type, CobA-type, and PduO-type enzymes (14). Genes encoding EutT-type enzymes are organized exclusively with genes encoding AdoCbl-dependent ethanolamine ammonia lyases and are presumed to function in support of ethanolamine degradation (19). CobA and PduO homologues are broadly distributed among *Archaea* and *Bacteria*, and enzymes distantly related to PduO are also found in *Caenorhabditis elegans*, mice, cows, and humans (14, 15, 19). Both the CobA and the PduO adenosyltransferases were originally identified in *Salmonella enterica* (7, 14). The CobA enzyme plays a role in the de novo synthesis of AdoCbl as well as the assimilation of exogenous corrinoid compounds (7, 25, 26). The PduO adenosyltransferase is encoded by the 1,2-propanediol utilization (*pdu*) operon (14). Its primary role is to supply cofactor for AdoCbl-dependent 1,2-propanediol degradation, and it is an integral component of an unusual polyhedral body proposed to mitigate aldehyde toxicity during this process (11). Bioinformatic analyses indicate that the PduO enzyme has two distinct domains since its N- and C-terminal regions (amino acids 1 to 185 and 186 to 336) align with complete proteins encoded by different genes. The N-terminal domain has 30% identity to the human adenosyltransferase and 27% identity to the *Thermoplasma acidophilum* adenosyltransferase and also aligns with a number of proteins whose functions have not been investigated experimentally (14, 15, 22). The C-terminal domain of PduO has significant similarity to 28 proteins of unknown function found in GenBank (expect value, $<7 \times 10^{-4}$),

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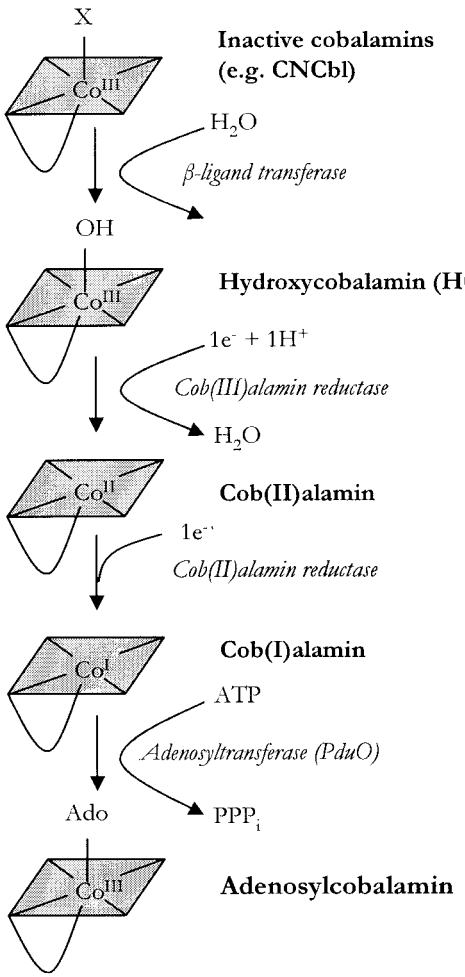

FIG. 1. Proposed pathway for the conversion of CNCbl to AdoCbl. This pathway is needed for the conversion of exogenous complex cobalamin precursors into AdoCbl as well as for the recycling of complex precursors generated intracellularly due to the breakdown of AdoCbl during catalysis.

but not to proteins of known function. Gene arrangement analyses indicate that homologues of the C-terminal domain of PduO function in AdoCbl-dependent 1,2-propanediol and glycerol degradation as well as in cobalamin-independent processes (14).

Here, we report a facile procedure for the purification of the *S. enterica* PduO adenosyltransferase and partially characterize the purified enzyme biochemically. Investigations of truncated versions of the PduO enzyme confirm that adenosyltransferase activity is associated with its N-terminal domain. Kinetic studies indicate that the PduO enzyme is sufficient to mediate AdoCbl synthesis in vivo and that the enzyme uses a ternary complex mechanism. In addition, PduO is shown to have stringent nucleotide specificity and triphosphate is established as a reaction product.

MATERIALS AND METHODS

Chemicals and reagents. Titanium (III) citrate was prepared as described previously (3). Hydroxycobalamin, sodium pyrophosphate, sodium triphosphate, and orthophosphate were from Sigma Chemical Company, St. Louis, Mo. Restriction enzymes and T4 DNA ligase were from New England Biolabs, Beverly, Mass. All other chemicals were from Fisher Scientific, Norcross, Ga.

Molecular techniques. Agarose gel electrophoresis was performed as described previously (16). Plasmid DNA was purified with QIAGEN (Chatsworth, Calif.) products according to the manufacturer's instructions. Following restriction enzyme digestion or PCR amplification, DNA was purified by phenol-chloroform extraction followed by ethanol precipitation or by use of QIAGEN gel extraction kits. Restriction digests were carried out according to standard protocols (16). For ligation of DNA fragments, T4 DNA ligase (New England Biolabs) was used according to the manufacturer's instructions. Bacteria were transformed by electroporation as described previously (14). Luria-Bertani (LB) medium containing antibiotic(s) was used to select for transformed cells, and single-colony isolates were used for analysis.

P22 transduction. Transductional crosses were performed as described previously with phage P22 HT105/1 *int*-201, a mutant phage that has high transducing ability (23). Transductants were selected with LB medium containing ampicillin (100 μg/ml). For the preparation of P22 transducing lysates from *S. enterica* TR6579, overnight cultures were grown with LB medium supplemented with 11 mM glucose and 11 mM galactose.

Protein techniques. Protein concentration was determined with Bio-Rad protein assay reagent according to the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with Bio-Rad Ready gels and Mini-protean II electrophoresis cells run at 200 V for 45 min with a Bio-Rad Power Pac 300. Following electrophoresis, proteins were stained with Coomassie brilliant blue R-250.

Cloning of truncated versions of the *pduO* gene for protein expression. DNA for cloning truncated versions of the *pduO* gene was obtained via PCR amplification of plasmid pMGS-2 (14) using forward primer 5′-GGAATTCAGATCT TATGGCGATTTATACCCGAAC-3′ and reverse primer 5′-GCCGCCAAGC TTTCAAATCTCTTCGCTGCTGATGTA-3′, 5′-GCCGCCAAGCTTTCACA GATAGCGCTTGCTAACTTC-3′, 5′-GCCGCCAAGCTTTCAGTCAACGAT GCTCACGACTAC-3′, or 5′-GCCGCCAAGCTTTCAAAAGGTGACCACTT TTCCCTG-3′. The C-terminal domain of the *pduO* gene was amplified with primers 5′-GGAATTCAGATCTTATGCACAGCAAGGAGACGACGC-3′ and 5′-GGAATTCAAGCTTGGTTTCGAGTTCAGAAGTATTC-3′. *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) was employed for the amplification. Amplified DNA was purified, restricted, and ligated to plasmid pTA925, a modified T7 expression vector (14) that had been previously digested with restriction enzymes BglII and HindIII, and gel purified. The ligation mixture was used to transform *Escherichia coli* DH5α. LB medium containing kanamycin (25 μg/ml) was used to select transformants. Plasmid DNA was purified from selected transformants and screened by digestion with BglII and HindIII. Plasmids releasing fragments of the expected sizes (280, 565, 700, 889, and 462 bp) were used to transform the expression strain *E. coli* BL21(DE3) RIL (Stratagene). The DNA sequence of each clone was verified, and the resulting strains (BE294 to BE298) were used for production of truncated versions of the PduO enzyme.For complementation studies, truncated versions of the *pduO* gene were subcloned from the expression vectors into pLAC22 by using BglII and HindIII restriction sites. Vector pLAC22 allows the expression of cloned genes to be very stringently regulated (29). The resulting plasmids were used to transform *S. enterica* TR6579 by electroporation. Then, P22 transduction was used to transfer the subclones into *S. enterica* BE121 (*cobA pduO*) (14), and the resulting strains (BE299 to BE303) were used for complementation studies.

Production of recombinant PduO and truncated versions. Protein expression strains were grown in 1-liter baffled Erlenmeyer flasks containing 400 ml of LB broth and 25-μg/ml kanamycin. A 2-ml LB overnight culture was used as the inoculum, and cultures were incubated at 30°C in a gyratory water bath (New Brunswick) with shaking at 250 rpm, until an optical density at 600 nm of 0.5 was attained. The expression of recombinant protein was induced by the addition of isopropyl-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM, and cultures were incubated at 30°C for an additional 3 h after IPTG addition. Both the full-length protein and its truncated version produced inclusion bodies when expressed at high levels.

Purification of recombinant PduO and PduO$^{1-185}$ enzymes. Cells from 400-ml cultures were collected by centrifugation for 15 min at 10,500 × *g* with a Beckman (Fullerton, Calif.) J2-HS centrifuge and a Beckman JA-10 rotor. The pellet was suspended in 3 ml of buffer A (50 mM potassium phosphate at pH 7.5, 20 mM sodium chloride, 1 mM Pefabloc protease inhibitor [Pentapharm, Ltd., Basel, Switzerland]) and lysed by single pass through a French press at 137.9 Mpa. Insoluble material was collected by centrifugation at 27,500 × *g* for 30 min with a Beckman JA-20 rotor. The supernatant was discarded, and the insoluble pellet was treated with Bacterial Protein Extraction Reagent II (B-PER II; Pierce, Rockford, Ill.) according to the manufacturer's "midi-prep" protocol with

the following modifications. The pellet was initially suspended in 5 ml of B-PER II, and following lysozyme treatment, 15 ml of 1:20 dilute B-PER II solution was added. The inclusion bodies were washed twice with 20 ml of 1:20 dilute B-PER II solution supplemented with 5 mM dithiothreitol and washed once with buffer A. The inclusion bodies were harvested by centrifugation and suspended in solubilization buffer [25 mM 2-(cyclohexylamino) ethanesulphonic acid (CHES) at pH 8.5, 1 mM dithiothreitol] to a final concentration of 5 mg/ml; solubilization was carried out overnight at 0°C. Insoluble material was separated from soluble protein by centrifugation at 27,500 × *g* for 10 min. The supernatant was recovered and filtered with a 0.22-μm-pore cellulose-acetate filter. The filtrate was applied to a 25-ml bed-volume phenyl Sepharose HP column (Amersham Biosciences, Uppsala, Sweden) that was equilibrated with 25 mM CHES at pH 8.5. PduO or PduO$^{1-185}$ were eluted with a linear gradient from 25 mM to 750 mM CHES at pH 8.5. Fractions containing the enzyme of interest were combined and concentrated with a Vivascience 10,000-molecular-weight-cutoff concentrator.

Partial purification of recombinant PduO and its truncated versions. For determination of the domain(s) required for adenosyltransferase activity, PduO and its truncated versions were partially purified by extraction of inclusion bodies using B-PER II followed by solubilization with CHES as described above for purification of recombinant PduO.

Adenosyltransferase assays. Adenosyltransferase assays were performed at 37°C and pH 8.0 with 50 μg of recombinant protein using the continuous spectrophotometric method described previously (14). Briefly, reaction mixtures were prepared in an anaerobic hood and contained 200 mM Tris-HCl at pH 8.0, 5 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM ATP, 0.05 mM HOCbl, and 1 mM titanium (III) citrate in a final volume of 2 ml. The rate of AdoCbl production was calculated by using the average of at least three determinations.

Kinetic analyses. The adenosyltransferase reaction has two substrates. Therefore, in kinetic analyses, one substrate was used in excess while the concentration of the other was varied. To determine the dependence of adenosyltransferase activity on ATP concentration, hydroxycobalamin was used at 0.05 mM (11 times the K_m), and the ATP concentration was varied from 0 to 0.2 mM. When the effect of cob(I)alamin concentration on reaction rate was determined, ATP was used at 0.4 mM (20 times the K_m) and cob(I)alamin was varied from 0 to 0.05 mM. Assays contained 50 μg of purified PduO or PduO$^{1-185}$, and initial reaction rates were measured. (Typically <0.5% of the substrate was consumed during the assay period.) Each experiment was repeated three times.

^{31}P NMR spectroscopy. The products of the PduO reaction were analyzed by ^{31}P nuclear magnetic resonance (NMR) spectroscopy. The adenosyltransferase assay was performed in an anaerobic test tube containing 200 mM Tris-HCl at pH 8.0, 5 mM KCl, 2.5 mM $MgCl_2$, 2.0 mM ATP, 2.0 mM HOCbl, 5 mM titanium (III) citrate, and 500 μg of PduO protein in a final volume of 5 ml. The reaction mixtures were incubated in the dark at 37°C for 2 h. Samples from the reaction mixture were transferred to 12-mm NMR tubes. EDTA was added to a final concentration of 75 mM, and 0.5 ml of 100% D_2O was added. ^{31}P NMR spectra of reference compounds and adenosyltransferase reaction products were obtained with a 7.0-T magnet (IFAS NMR Facility, University of Florida, Gainesville) and a modified Nicolet NT300 spectrometer (6) at the following settings: frequency, 121.469 MHz; excitation pulse width, 15 μs; pulse repetition delay, 6 s; and spectral width, 10 kHz. Spectra were Fourier transformed with Felix analytical software (Molecular Simulations, Inc., San Diego, Calif.). Chemical shifts were referenced to an inorganic phosphoric acid internal standard, which was set to 0.0 ppm.

RESULTS AND DISCUSSION

Purification of PduO adenosyltransferase. The *S. enterica* PduO protein was produced at high levels in *E. coli*, using a modified T7 expression vector as previously described (14). When produced in this manner, the PduO protein is found in the insoluble fraction of cell extracts but is catalytically active (14). The PduO adenosyltransferase was solubilized in an active form by using CHES buffer, which has detergent-like properties, and purified to near homogeneity by hydrophobic interaction chromatography. Following the phenyl Sepharose chromatography, only fractions of the highest purity were saved for further study. The progress of the purification was monitored by SDS-PAGE and enzymatic assay (Table 1). Following staining with Coomassie brilliant blue, the purified PduO protein appeared homogenous (not shown). The yield was 23%, and the fold purification was 1.4. The relatively low fold purification is a consequence of the fact that the inclusion bodies were composed primarily of the PduO enzyme. The finding that the yield was >100% following solubilization with CHES suggests that the activity of PduO is inhibited somewhat by incorporation into inclusion bodies. Overall, the purification allowed the isolation of 20 mg of purified PduO in a 2-day period.

TABLE 1. Purification of the PduO adenosyltransferase used in this study

Purification step	Total protein (mg)	Sp act. (nmol min^{-1} mg^{-1})	Total activity (nmol min^{-1})	Yield (%)	Purification (fold)
Inclusion body isolation	120	194	23,280	100	1
Solubilization with CHES	120	248	29,760	128	1.3
Phenyl Sepharose chromatography[a]	20	262	5,240	23	1.4

[a] In the phenyl sepharose chromatography step, only the fractions of highest purity were pooled and used for further study. Typically about 80 to 90% of the total activity was recovered from the phenyl Sepharose column, but the majority of the eluate contained a minor contaminant and was discarded.

During the course of our studies, a variety of conditions were tested for solubilization of PduO from inclusion bodies. Only CHES and glycylglycine buffers were effective. Other detergents commonly used for protein solubilization were not useful. Prior studies have shown that the PduO enzyme is part of a polyhedral body involved in 1,2-propanediol degradation that consists of at least 15 different polypeptides (11). This suggests that when it is expressed in the recombinant form, PduO self-associates in lieu of interacting with polyhedral body proteins. This raises the possibility that CHES and glycylglycine buffers may be generally useful for solubilizing a variety of proteins normally found as part of prokaryotic polyhedral bodies or in other multiprotein complexes.

Linearity and requirements of the PduO reaction. The effect of enzyme concentration on the rate of adenosyltransfer was examined. Adenosyltransferase activity was directly proportional to the PduO concentration in the range of 10 to 200 μg of protein (rate, 197 nmol min^{-1} mg^{-1}; and $R^2 = 0.9928$). The omission of PduO, ATP, HOCbl, or titanium (III) citrate—the reducing agent that converts HOCbl to cob(I)alamin—eliminated measurable activity.

Divalent metal ion requirements of the PduO reaction. Adenosyltransferase assays were performed under standard conditions, except that Mg^{2+} was omitted or replaced with other divalent metal ions. Without added divalent metal, adenosyltransferase activity of 91 nmol min^{-1} mg^{-1} (32%) was observed. The highest activities were obtained with Mg^{2+}, Mn^{2+}, or Co^{2+} (285, 277, or 266 nmol of AdoCbl min^{-1} mg^{-1}, respectively). Added Ca^{2+} had little effect on activity (81 nmol min^{-1} mg^{-1}), and Cd^{2+}, Zn^{2+}, Ni^{2+}, or Cu^{2+} abolished measurable activity.

The divalent metal ion requirements of PduO are similar to those reported for the *T. acidophilum* adenosyltransferase, which was also most active with Mg^{2+} (22). However, they are

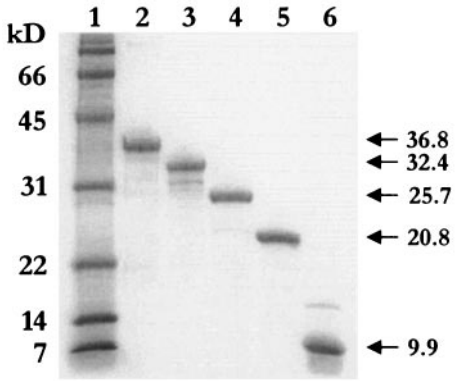

FIG. 2. SDS-PAGE analysis of solubilized truncated versions of the PduO protein. Each lane contains 4 μg of recombinant protein. Lane 1, molecular mass markers; lane 2, PduO; lane 3, PduO$^{1-293}$; lane 4, PduO$^{1-230}$; lane 5, PduO$^{1-185}$, lane 6, PduO$^{1-90}$. PduO and its truncated versions were partially purified as described in Materials and Methods.

somewhat different from results obtained with the CobA and *C. tetanomorphum* enzymes, both of which showed maximal activity when Mn^{2+} was included in the reaction mixture and partial activity with Mg^{2+} or Co^{2+} (25, 27).

The N-terminal domain of PduO has adenosyltransferase activity. Bioinformatic analyses indicated that the *S. enterica* PduO protein has two domains (14). The N-terminal domain corresponds to amino acids 1 to 185, and the C-terminal domain is composed of amino acids 186 to 336. To investigate the functions of these domains, a series of truncated versions of the PduO protein were produced in *E. coli*, partially purified, and assayed for adenosyltransferase activity. The polypeptides produced were composed of the first 90, 185, 230, and 293 amino acids of the PduO protein (PduO$^{1-90}$, PduO$^{1-185}$, PduO$^{1-230}$, and PduO$^{1-293}$). Each protein was partially purified from inclusion bodies by treatment with B-PER II followed by solubilization with CHES buffer, and SDS-PAGE indicated that each protein was 80 to 90% pure (Fig. 2). Enzyme assays demonstrated that PduO$^{1-185}$, PduO$^{1-230}$, and PduO$^{1-193}$ (each of which has deletions of all or part of the C-terminal domain) had adenosyltransferase activity, with turnover numbers similar to that of the full-length PduO protein (Table 2). The specific activities of these truncated proteins are higher than that of the full-length protein because of their reduced molecular masses. In contrast, disruption of the N-terminal domain (PduO$^{1-90}$) abolished measurable adenosyltransferase activity.

TABLE 2. Adenosyltransferase activities of truncated versions of PduO[a]

Protein	Size (amino acids)	Approx mol mass (Da)	Adenosyltransferase activity (nmol min^{-1} mg^{-1})	k_{cat} (min^{-1})
PduO$^{1-90}$	1–90	9,908	ND[b]	ND
PduO$^{1-185}$	1–185	20,889	433	9.04
PduO$^{1-230}$	1–230	25,760	366	9.42
PduO$^{1-293}$	1–293	32,489	284	9.23
PduO	1–336	36,812	262	9.64

[a] PduO and its truncated versions were partially purified as described in Materials and Methods.
[b] ND, not detectable under conditions used.

Thus, in vitro studies indicated that adenosyltransferase activity of PduO is associated with its N-terminal domain.

To test for adenosyltransferase activity in vivo, the *pduO* gene and each truncated version were individually expressed in *S. enterica* strain BE121 (*pduO cobA*), which is unable to degrade 1,2-propanediol due to an adenosyltransferase deficiency (14). PduO$^{1-185}$, PduO$^{1-230}$, and PduO$^{1-293}$ restored the ability of BE121 to degrade 1,2-propanediol; however, the PduO$^{1-90}$ was unable to complement this strain. Since disruption of the N-terminal domain of PduO resulted in the loss of adenosyltransferase activity in vitro and in vivo, whereas disruption of the C-terminal domain had little effect, we conclude that the adenosyltransferase activity is associated with the N-terminal domain.

Kinetic measurements. In vitro assays indicated that both PduO and PduO$^{1-185}$ followed Michaelis-Menten kinetics with respect to both ATP and cob(I)alamin. Using the Eadie-Hofstee method, the K_m values of PduO and PduO$^{1-185}$ were determined to be 19.8 and 19.3 μM for ATP and 4.5 and 4.3 μM for cob(I)alamin, respectively. The V_{max}s were 243 nmol min^{-1} mg^{-1} for PduO and 452 nmol min^{-1} mg^{-1} for PduO$^{1-185}$. The turnover numbers (k_{cat}) for PduO and PduO$^{1-185}$ were similar (9.64 and 9.04 min^{-1}), indicating that the higher V_{max} of PduO$^{1-185}$ was the result of its lower molecular mass but otherwise was not related to deletion of the C-terminal domain.

The kinetic constants for PduO are comparable to those previously reported for bacterial adenosyltransferase enzymes, for which specific activities range from 53 to 619 nmol min^{-1} mg^{-1} and K_m values ranged from 2.8 to 110 μM for ATP and from 3 to 5.2 μM for cob(I)alamin (22, 25, 27). These values are appropriate to the physiological role of adenosyltransferase enzymes, since cellular levels of cobalamin are typically 1 to 10 μM in prokaryotic organisms and ATP concentrations are in the low millimolar range (1, 24). Thus, the kinetic properties of PduO indicate that this enzyme is sufficient to meet cellular needs for AdoCbl synthesis.

Specificity for the donor nucleotide. The abilities of PduO and PduO$^{1-185}$ to use nucleotides other than ATP as the donor molecule for the β ligand of cobalamin were measured. PduO and PduO$^{1-185}$ showed no measurable catalytic activity when adenine, adenosine, AMP, ADP, CTP, GTP, UTP, or 2′-deoxyadenosine was used in place of ATP, even after prolonged incubation (up to 30 min) at 37°C. The PduO adenosyltransferase displayed partial activity (21%) when 2′-deoxyadenosine-5′-triphosphate (dATP) was used in place of ATP (44 compared to 210 nmol min^{-1} mg^{-1}).

The findings that PduO has stringent nucleotide specificity and partial activity (21%) with dATP are similar to results recently reported for the adenosyltransferase from the archaeon *T. acidophilum* (22). The archaeal enzyme is 177 amino acids in length and 27% identical to the N-terminal domain of the PduO enzyme. It differs from the PduO enzyme in that it lacks a C-terminal domain and is not known to be associated with a polyhedral body (22). Nonetheless, these results are suggestive that the PduO and *T. acidophilum* enzymes may bind ATP in a similar manner.

In contrast to the PduO and *T. acidophilum* adenosyltransferases, the CobA enzyme has a relatively low nucleotide specificity and is inactive with dATP under standard assay condi-

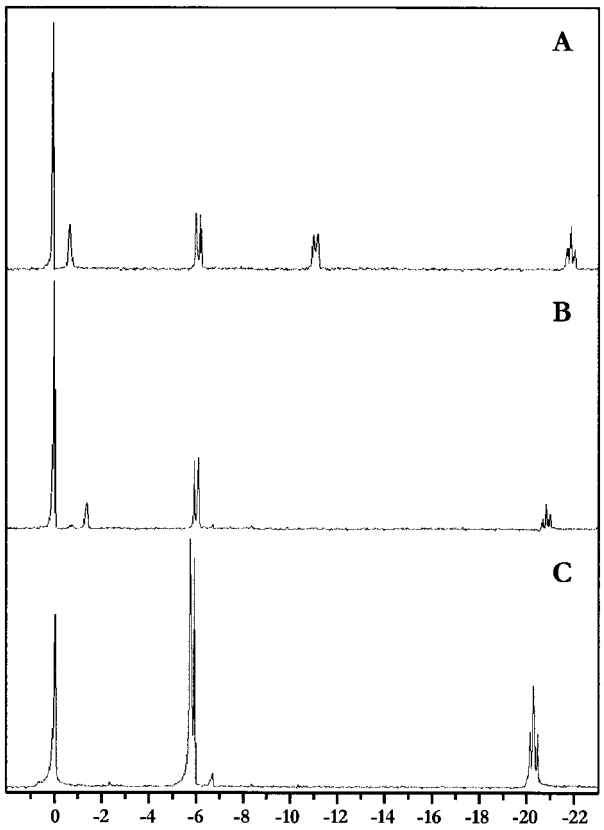

FIG. 3. ^{31}P NMR spectra of a PduO reaction. All chemical shifts are referenced to H_3PO_4, which is set to 0 ppm. (A) Reaction mixture prior to addition of PduO containing 2 mM ATP and 2 mM HOCbl; chemical shifts observed at −6.2 ppm, −11.2 ppm, and −21.8 ppm correspond to the γ-, α-, and β-phosphates of ATP, respectively; the singlet at −0.7 ppm corresponds to the phosphate of the nucleotide loop of HOCbl. (B) Complete reaction after 2 h of incubation with 500 μg of purified PduO. (C) Inorganic triphosphate standard (0.1 M; center phosphate, triplet centered at −20.3 ppm; outer phosphates, doublet centered at −5.8 ppm).

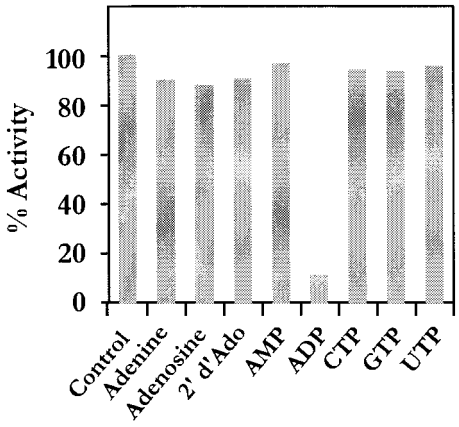

FIG. 4. Inhibition of PduO by nucleotides other than ATP and adenine-containing compounds. Adenosyltransferase assays were performed with 25 μg of purified PduO protein. The concentration of ATP used was 50 μM, and the concentration of the inhibitor was 100 μM. The chart shows the percentage of adenosyltransferase remaining in the presence of inhibitor. 2′ d'Ado, 2′-deoxyadenosine.

tions (8). Previously the three-dimensional structure of the CobA adenosyltransferase complexed with MgATP was determined (2). The CobA protein contains a modified Walker A motif, defined by residues Gly^{36} to Thr^{43} (GNGKGKTT), which associates with the α-, β-, and γ-phosphates of ATP via extensive hydrogen bonding (2). Fewer hydrogen bonds are found between the adenosine moiety and the CobA protein, which may explain its relatively low nucleotide specificity (2, 8). In addition, the γ-phosphate and 2′-OH of ATP form an intramolecular hydrogen bond which is proposed to be critical for catalysis, accounting for the lack of activity of CobA with dATP (8). In contrast, the sequences of the PduO and *T. acidophilum* adenosyltransferases have no distinguishable P-loop or Walker motif, and both have partial activity with dATP, indicating an alternative mode of ATP binding as well as possible differences in the catalytic mechanisms (22).

Triphosphate is the product of the PduO adenosyltransferase reaction. Prior studies demonstrated that AdoCbl was a product of the PduO adenosyltransferase reaction, but the fate of the triphosphate moiety of ATP was not determined (14). To address this question, ^{31}P NMR spectra were determined for the substrates and products of the PduO reaction (Fig. 3). Fig. 3A shows the ^{31}P NMR spectrum of an adenosyltransferase reaction mixture prior to addition of the PduO enzyme. The triplet centered at −21.8 ppm corresponds to the β-phosphate of ATP; the two doublets centered at −6.2 ppm and −11.2 ppm correspond to the γ- and α-phosphates of ATP, respectively. The singlet at −0.7 ppm results from the phosphate group that is in the nucleotide loop of the cobalamin. Fig. 3B shows the complete reaction mixture after 2 h of incubation at 37°C with 500 μg of purified PduO. The doublet corresponding to the α-phosphate of ATP (−11.2 ppm) is absent, indicating that the reaction went to completion. The triplet centered at −20.7 ppm and the doublet centered at −6 ppm of the complete reaction mixture correlate well with the triplet at −20.3 ppm and the doublet at −5.8 ppm of the sodium triphosphate standard (Fig. 3C). The singlet at −1.4 ppm corresponds to the phosphate group of the nucleotide loop of AdoCbl, since for the authentic AdoCbl standard, this phosphate had a chemical shift of −1.4 ppm. The shift of this resonance upfield from −0.7 ppm to −1.4 ppm was likely caused by the change in conformation of the corrin ring that occurs when the 5′-deoxyadenosyl group is added to the β-axial position. It is also of note that the spectrum of the completed reaction (Fig. 3B) lacked a singlet at −6.7 ppm which is characteristic of pyrophosphate, demonstrating that this compound was not a major reaction product. Hence, these results indicate that triphosphate and AdoCbl are the products of the PduO reaction and that significant hydrolysis of the triphosphate to orthophosphate and pyrophosphate did not occur.

Thus, the reaction products of PduO appear similar to those reported for the *S. enterica* CobA protein (8) and the *C. tetanomorphum* adenosyltransferase (17), but are different from those of the *Propionibacterium shermanii* enzyme, which has been reported to release orthophosphate and pyrophosphate (5). Note that the genes for the *C. tetanomorphum* and *P. shermanii* adenosyltransferases have not been identified, and the phosphate product of the *T. acidophilum* enzyme has not been reported (22).

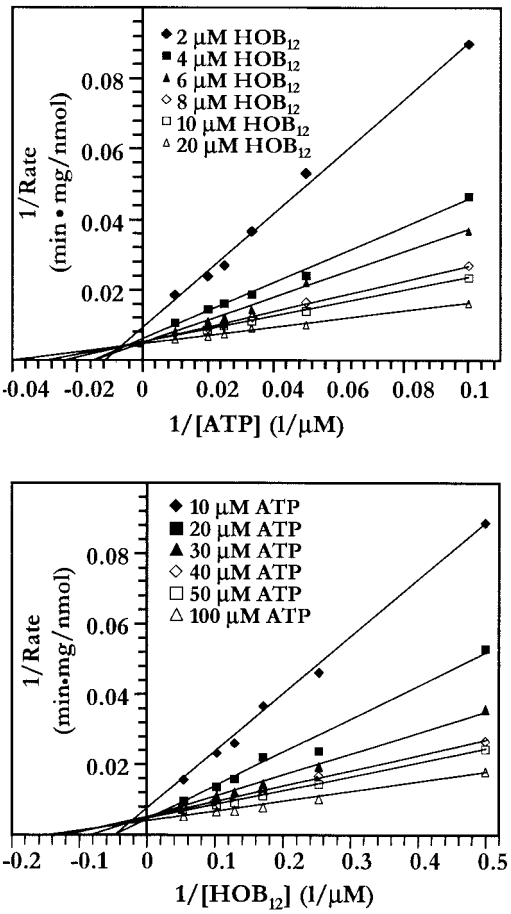

FIG. 5. Double-reciprocal plots showing the effect of varying both ATP and HOCbl concentrations on the rate of adenosyltransferase activity. Adenosyltransferase assays were performed with 25 μg of purified PduO protein. HOCbl (HOB_{12}) was held constant at 2, 4, 6, 8, 10, or 20 μM, while the concentration of ATP was varied from 10 to 100 μM. The experiment was repeated three times with similar results, and the average of the three experiments is shown.

Inhibition of the PduO reaction by nucleotides other than ATP. To test for possible inhibitory effects of nucleotides other than ATP on the PduO reaction, adenosyltransferase assays were conducted that contained 50 μM ATP and 100 μM competing nucleotide. PduO retained 95% activity when AMP, CTP, GTP, or UTP was added to reaction mixtures (Fig. 4). When adenine, adenosine, or 2′-deoxyadenosine was included, 90% activity was retained. However, only 10% activity remained when ADP was included, and kinetic analyses in which the concentrations of both ATP and ADP were varied indicated a K_i of 4.9 μM (data not shown). The finding that ADP is inhibitory whereas AMP is not suggests a role for the β-phosphate in the binding of ATP to the PduO adenosyltransferase.

Phosphate inhibition. The effects of orthophosphate, pyrophosphate, and triphosphate on the PduO reaction were also investigated. Orthophosphate had no effect on the adenosyltransferase activity of PduO when used at concentrations up to 4 mM (ATP/phosphate ratio of 1:10). However, at 4 mM pyrophosphate or triphosphate, PduO had 29 or 71% of the activity measured in the standard assay, respectively (65 or 165 nmol min^{-1} mg^{-1} compared to 222 nmol min^{-1} mg^{-1}). In contrast, CobA showed severe product inhibition by triphosphate, with only 15% activity remaining when the ATP/triphosphate ratio was 1:2.5 (8). These results show that the interaction of triphosphate is relatively weak with PduO compared to CobA. This suggests that CobA binds more tightly to the triphosphate moiety of ATP than does PduO, which is consistent with the studies described above that indicate these two enzymes bind ATP differently.

Evidence for a ternary complex. In order to determine whether a covalent intermediate existed in the reaction mechanism, the effects of various concentrations of both ATP and HOCbl on the rate of adenosyltransfer were examined. For these studies, HOCbl was used at a concentration of 2, 4, 6, 8, 10, or 20 μM, as the ATP concentration was varied from 10 to 100 μM, and the data were analyzed by the double-reciprocal method of Lineweaver and Burk (Fig. 5). Results showed that both the slope and the *x* intercept of the Lineweaver-Burk plot vary with the concentration of either ATP or HOCbl. This suggests that PduO employs a ternary complex reaction mechanism rather than a substituted enzyme mechanism. Many ATP-dependent enzymes employ random ternary complex mechanisms during catalysis, and this may be the case for the PduO adenosyltransferase, although these studies do not rule out an ordered ternary complex mechanism.

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REFERENCES

1. **Banerjee, R. (ed.).** 1999. Chemistry and biochemistry of B_{12}. John Wiley and Sons, New York, N.Y.
2. **Bauer, C. B., M. V. Fonseca, H. M. Holden, J. B. Thoden, T. B. Thompson, J. C. Escalante-Semerena, and I. Rayment.** 2001. Three-dimensional structure of ATP:corrinoid adenosyltransferase from *Salmonella typhimurium* in its free state, complexed with MgATP, or complexed with hydroxycobalamin and MgATP. Biochemistry **40:**361–374.
3. **Bobik, T. A., and R. S. Wolfe.** 1989. Activation of formylmethanofuran synthesis in cell extracts of *Methanobacterium thermoautotrophicum*. J. Bacteriol. **171:**1423–1427.
4. **Brady, R. O., and H. A. Barker.** 1961. The enzymatic synthesis of cobamide coenzymes. Biochem. Biophys. Res. Commun. **4:**464–468.
5. **Brady, R. O., E. G. Castanera, and H. A. Barker.** 1962. The enzymatic synthesis of cobamide coenzymes. J. Biol. Chem. **237:**2325–2332.
6. **Buszko, M. L., and D. C. Wang.** 1996. A world-wide web interface to an NMR spectrometer. Appl. Magn. Reson. **12:**95–101.
7. **Escalante-Semerena, J. C., S.-J. Suh, and J. R. Roth.** 1990. *cobA* function is required for both de novo cobalamin biosynthesis and assimilation of exogenous corrinoids in *Salmonella typhimurium*. J. Bacteriol. **172:**273–280.
8. **Fonseca, M. V., N. R. Buan, A. R. Horswill, I. Rayment, and J. C. Escalante-Semerena.** 2002. The ATP:Co(I)rrinoid adenosyltransferase (CobA) enzyme of *Salmonella enterica* requires the 2′-OH group of ATP for function and yields inorganic triphosphate as its reaction byproduct. J. Biol. Chem. **277:** 33127–33131.
9. **Fonseca, M. V., and J. C. Escalante-Semerena.** 2000. Reduction of cob (III)alamin to cob(II)alamin in *Salmonella enterica* serovar Typhimurium LT2. J. Bacteriol. **182:**4304–4309.
10. **Friedmann, H. C.** 1975. Biosynthesis of corrinoids, p. 75–103. *In* B. M. Babior (ed.), Cobalamin. John Wiley and Sons, New York, N.Y.
11. **Havemann, G. D., and T. A. Bobik.** 2003. Protein content of polyhedral organelles involved in coenzyme B_{12}-dependent degradation of 1,2-propanediol in *Salmonella enterica* serovar Typhimurium LT2. J. Bacteriol. **185:**5086–5095.
12. **Honda, S., T. Toraya, and S. Fukui.** 1980. In situ reactivation of glycerol-inactivated coenzyme B_{12}-dependent enzymes, glycerol dehydratase and diol dehydratase. J. Bacteriol. **143:**1458–1465.

13. **Huennekens, F. M., K. S. Vitols, K. Fujii, and D. W. Jacobsen.** 1982. Biosynthesis of the cobalamin coenzymes. *In* D. Dolphin (ed.), B_{12}. John Wiley and Sons, New York, N.Y.
14. **Johnson, C. L. V., E. Pechonick, S. D. Park, G. D. Havemann, N. A. Leal, and T. A. Bobik.** 2001. Functional genomic, biochemical, and genetic characterization of the *Salmonella pduO* gene, an ATP:cob(I)alamin adenosyltransferase gene. J. Bacteriol. **183:**1577–1584.
15. **Leal, N. A., S. D. Park, P. E. Kima, and T. A. Bobik.** 2003. Identification of the human and bovine ATP:Cob(I)alamin adenosyltransferase cDNAs based on complementation of a bacterial mutant. J. Biol. Chem. **278:**9227–9234.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Peterkofsky, A., and H. Weissbach.** 1963. Release of inorganic tripolyphosphate from adenosine triphosphate during vitamin B_{12} coenzyme biosynthesis. J. Biol. Chem. **238:**1491–1497.
18. **Pezacka, E.** 1993. Identification of and characterization of two enzymes involved in the intracellular metabolism of cobalamin. Cyanocobalamin β-ligand transferase and microsomal cob(III)alamin reductase. Biochem. Biophys. Acta **1157:**167–177.
19. **Rodionov, D. A., A. G. Vitreschak, A. A. Mironov, and M. S. Gelfand.** 2003. Comparative genomics of the vitamin B_{12} metabolism and regulation in prokaryotes. J. Biol. Chem. **278:**41148–41159.
20. **Roth, J. R., J. G. Lawrence, and T. A. Bobik.** 1996. Cobalamin (coenzyme B_{12}): synthesis and biological significance. Annu. Rev. Microbiol. **50:**137–181.
21. **Saido, H., F. Watanabe, Y. Tamura, Y. Funae, S. Imaoka, and Y. Nakano.** 1993. Mitochondrial NADPH-linked aquacobalamin reductase is distinct from the NADPH-linked enzyme from microsomal membranes from rat liver. J. Nutr. **123:**1868–1874.
22. **Saridakis, V., A. Yakunin, X. Xu, P. Anandakumar, M. Pennycooke, J. Gu, F. Cheung, J. M. Lew, R. Sanishvili, A. Joachimiak, C. H. Arrowsmith, D. Christendat, and A. M. Edwards.** 2004. The structural basis for methylmalonic aciduria: the crystal structure of archael ATP:cobalamin adenosyltransferase. J. Biol. Chem. **279:**23646–23653.
23. **Schmieger, H.** 1971. A method for detection of phage mutants with altered transducing ability. Mol. Gen. Genet. **110:**378–381.
24. **Schneider, Z., and A. Stroinski.** 1987. Comprehensive B_{12}: chemistry, biochemistry, nutrition, ecology, medicine. De Gruyter, Berlin, Germany.
25. **Suh, S.-J., and J. C. Escalante-Semerena.** 1995. Purification and initial characterization of the ATP:corrinoid adenosyltransferase encoded by the *cobA* gene of *Salmonella typhimurium*. J. Bacteriol. **177:**921–925.
26. **Suh, S. J., and J. C. Escalante-Semerena.** 1993. Cloning, sequencing and overexpression of *cobA*, which encodes ATP:corrinoid adenosyltransferase in *Salmonella typhimurium*. Gene **129:**93–97.
27. **Vitols, E., G. A. Walker, and F. M. Huennekens.** 1966. Enzymatic conversion of vitamin B_{12s} to a cobamide coenzyme, α-(5,6-dimethylbenzimidazolyl) deoxyadenosylcobamide (adenosyl-B_{12}). J. Biol. Chem. **241:**1455–1461.
28. **Walker, G. A., S. Murphy, and F. M. Huennekens.** 1969. Enzymatic conversion of vitamin B_{12a} to adenosyl-B_{12}: evidence for the existence of two separate reducing systems. Arch. Biochem. Biophys. **134:**95–102.
29. **Warren, J. W., J. R. Walker, J. R. Roth, and E. Altman.** 2000. Construction and characterization of a highly regulable expression vector, pLAC11, and its multipurpose derivatives, pLAC22 and pLAC33. Plasmid **44:**138–151.
30. **Watanabe, F., Y. Nakano, S. Maruno, N. Tachikake, Y. Tamura, and S. Kitaoka.** 1989. NADH- and NADPH-linked aquacobalamin reductases occur in both mitochondrial and microsomal membranes of rat liver. Biochem. Biophys. Res. Commun. **165:**675–679.
31. **Watanabe, F., Y. Nakano, N. Tachikake, S. Kitaoka, Y. Tamura, and H. Yamanaka.** 1991. Occurrence and subcellular location of NADH- and NADPH-linked aquacobalamin reductases in human liver. Int. J. Biochem. **23:**531–533.
32. **Watanabe, F., Y. Oki, Y. Nakano, and S. Kitaoka.** 1987. Purification and characterization of aquacobalamin reductase (NADPH) from *Euglena gracilis*. J. Biol. Chem. **262:**11514–11518.
33. **Weissbach, H., B. G. Redfield, and A. Peterkofsky.** 1962. Biosynthesis of the B_{12} coenzyme: requirements for release of cyanide and spectral changes. J. Biol. Chem. **237:**3217–3222.