Structural Characterization of the Active Site of the PduO-Type ATP:Co(I)rrinoid Adenosyltransferase from Lactobacillus reuteri*

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The three-dimensional crystal structure of the PduO-type corrinoid adenosyltransferase from Lactobacillus reuteri (LrPduO) has been solved to 1.68-Å resolution. The functional assignment of LrPduO as a corrinoid adenosyltransferase was confirmed by in vivo and in vitro evidence. The enzyme has an apparent $K_m$ of 2.2 μM and $K_{m\text{cobalamin}}$ of 0.13 μM and a $k_{\text{cat}}$ of 0.025 s$^{-1}$. Co-crystallization of the enzyme with Mg-ATP resulted in well-defined electron density for an N-terminal loop that had been disorder in other PduO-type enzyme structures. This newly defined N-terminal loop makes up the lower portion of the enzyme active site with the other half being contributed from an adjacent subunit. These results provide the first detailed description of the enzyme active site for a PduO-type adenosyltransferase and identify a unique ATP binding motif at the protein N terminus. The molecular architecture at the active site offers valuable new insight into the role of various residues responsible for the human disease methylmalonic aciduria.

B12 (cobalamin, Cbl)$^7$ is an essential nutrient for animals, lower eukaryotes, and prokaryotes, but is synthesized exclusively by prokaryotes (1). Adenosylation of the corrin ring of Cbl generates coenzyme B12 (adenosylcobalamin, AdoCbl), an essential cofactor used by enzymes that catalyze intramolecular rearrangements (2−4), deaminations (5), dehydrations (6), reductions (7, 8), and reductive dehalogenations (9). Corrinoid adenosyltransferases play a key role in the biosynthesis of AdoCbl by covalently attaching the 5'-deoxyadenosyl moiety from ATP to the Co(I) ion of the corrin ring of Cbl (10, 11). These enzymes generate a biologically unique, labile cobalt-carbon bond that is the source of the unusual chemistry associated with the B12 cofactor. As such, understanding the mechanistic strategies of those enzymes involved in the formation of this carbon–metal bond is of considerable interest. In Salmonella enterica, three separate cob(I)alamin adenosyltransferases have been identified: CobA, PduO, and EutT. All cob(I)alamin adenosyltransferases identified to date belong to one of these three distinct families. CobA is the housekeeping enzyme of S. enterica and is involved in the anaerobic de novo synthesis of AdoCbl. The structure and function of this enzyme has been well characterized (12–16). The PduO and EutT enzymes, meanwhile, assimilate existing Cbl into AdoCbl. Both PduO and EutT are encoded within large, discrete operons of S. enterica where they play specialized roles in the catabolism of 1,2-propanediol or ethanolamine (17–19). Despite its specialized role in S. enterica, the PduO-type enzyme is the most widely distributed of these adenosyltransferases with homologues identified in species of archaeotes and prokaryotes, as well as in many eukaryotes, ranging from yeast to humans (19). In animals, PduO is the only cob(I)alamin adenosyltransferase enzyme available for the assimilation of dietary cobalamins into coenzyme B12.

Humans, who lack the ability to synthesize Cbl de novo, produce AdoCbl from reduced Cbl through a PduO-type adenosyltransferase (19−21). Patients with malfunctions in this enzyme suffer from methylmalonic aciduria and metabolic ketoacidosis (22). Initial biochemical, functional, and structural characterization of PduO-type corrinoid adenosyltransferases has recently been reported, including a high-resolution three-dimensional crystal structure of the PduO-type enzyme from the archaeon Thermoplasma acidophilum (23), and two additional homologous structures deposited in the RCSB protein data bank (Bacillus subtilis Yqyk, 1RTY; and a putative PduO from Mycobacterium tuberculosis, 2G2D). These structures reveal that the enzyme is a trimer with each subunit composed of a five helix–bundle. Unfortunately, the absence of bound substrates in these crystal structures has precluded identification of
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EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—To generate an N-terminal (His)_{6}-tagged recombinant construct of PduO, the L. reuteri pduO gene (GenBank accession numberAY780645) was PCR-amplified from L. reuteri strain CRL1098 genomic DNA isolated as described (27). The primers used for amplification were 5’-CGGGATCCGCTGAAGATTTATA-CAAAAAATGG-3’ (forward), and 5’-GGAATTCCTAGGCGAAAACGTCTTTACTGTT-3’ (reverse). The PCR fragment was restriction-digested with BamHI and EcoRI and ligated into the pET-28b vector (EMD Biosciences, Inc.) generating the expression plasmid pPDF19 (Table 1), which expresses the recombinant PduO protein with a 35-amino acid N-terminal tag. To generate a recombinant protein with a rTEV protease-cleavable N-terminal (His)_{6} tag, the L. reuteri pduO gene was PCR-amplified from plasmid pPDF19. The primers used for the amplification were 5’-AAAAAAACCATGTTGAAGATTTATA-TATACAAAATGGTGATAAGGGC-3’ (forward) and 5’-TTTTTTCGCGCGCGTTAGCGAAAAAC-3’ (reverse). The PCR fragment was cut with Ncol and NotI and was ligated into vector pTEV3 (Table 1) yielding plasmid pPDFU22. The pPDFU22 vector directs the synthesis of a recombinant N-terminally tagged protein with the TEV protease cleavage site four residues upstream of the first amino acid of the LrPduO protein. The N-terminal amino acid sequence of this construct is MSYYHHHHHHHDYDIPTSENLYFQGASAPM1V2 . . . where the location of the TEV protease cleavage site is underlined.

Strains, Media, and Chemicals—Bacterial strains and plasmids used in this study are listed in Table 1. Chemicals were purchased from Sigma. Lysogenic broth (LB) (28, 29) was used as rich medium to propagate bacteria nonselectively. Vogel and Bonner’s no-carbon E (NCE) minimal medium (30, 31) was used to assess behavior was monitored at 650 nm using a Bio-Tek EL808 96-well plate reader. Each well contained 198 l of fresh NCE medium that was inoculated with 2 l of an overnight culture of the strain of interest grown in LB medium; each strain was analyzed in triplicate. Plates (Becton Dickinson) were incubated under aerobic conditions at 37 °C over a 24-h period.

LrPduO Protein Production and Purification—Plasmids were transformed into Escherichia coli strain BL21(ADE3) for overexpression. Strains were grown at 37 °C with shaking in 1 liter of LB medium supplemented with kanamycin (25 µg/ml). After the culture reached an optical density (OD_{650}) of 0.6 – 0.7, synthesis of the phage T7 RNA polymerase

### Table 1: Strains and plasmids

| Strains | Source or Ref. |
|---------|---------------|
| E. coli (JE3992) | New England Biolabs |
| S. enterica | K. Sanderson via J. Roth |
| ECE683 | Escalante-Semerena Laboratory Collection |
| JE1293 | Escalante-Semerena Laboratory Collection |
| JE8740 | Escalante-Semerena Laboratory Collection |
| JE8741 | Escalante-Semerena Laboratory Collection |
| JE8742 | Escalante-Semerena Laboratory Collection |

### Notes

a Derivatives of S. enterica serovar Typhimurium strain LT2.

b CERELA stock collection, B12 producer.

c HEL stock collection, B12 producer.
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enzyme was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. Cells were grown for an additional 2 h at 37 °C with shaking, and harvested by centrifugation at 12,000 × g with a Beckman/Coulter Avanti J-25i centrifuge equipped with a JLA-16.250 rotor. The cell pellet was frozen at −80 °C until used. For protein purification, the pellets were thawed and re-suspended in 20 ml of Tris-HCl buffer (0.1 M, pH 8.0 at 4 °C) containing the protease inhibitor phenylmethanesulfonyl fluoride (0.8 mM), imidazole (20 mM), and NaCl (0.5 M). Cells were broken using a French pressure cell (1.03 × 107 kPa); three passages ensured >99% breakage. Cell debris was separated from soluble proteins by centrifugation at 4 °C for 45 min at 45,000 × g. The resulting supernatant was filtered (0.45 μm; Nalgene), and proteins were resolved on a 5-ml HiTrap FF column (Amersham Biosciences). Proteins were desorbed from the column with a linear gradient of Tris-HCl buffer (0.1 M, pH 8.0 at 4 °C) containing NaCl (0.5 M), EDTA, 2 mM), buffer B (buffer A lacking EDTA), and buffer C (Tris-HCl (10 mM, pH 8 at 4 °C) containing NaCl (0.3 M) and Mg-ATP (3 mM)). Final protein purity was assessed with Fotodyne’s FOTO/Eclipse® Electronic Documentation & Analysis System, including software packages FOTO/Analyst® PC Image v5.0 and TotalLab-abTM one-dimensional gel analysis v2003 from NonLinear Dynamics, Ltd. Purified LrPduO protein was estimated to be >99% homogeneous and gave a yield of 40 mg from 1 liter of broth culture. This highly purified LrPduO protein was flash-frozen in liquid nitrogen in 40-μl droplets and stored at −80 °C until used. LrPduO protein with a TEV cleavable, N-terminal poly-His tag was overproduced from plasmid pPDU22 and initially purified as described above. Prior to dialysis, purified rTEV protease (34–36) was mixed with LrPduO in a 1:50 rTEV:LrPduO molar ratio, and incubated at room temperature for 3 h. The mixture was dialyzed overnight against buffer D (buffer A containing imidazole, 10 mM), followed by a 4-h dialysis at 4 °C against buffer E (Tris-HCl, 0.1 M, pH 8 at 4 °C containing NaCl, 0.5 M, and imidazole, 10 mM). The rTEV:LrPduO protein mixture was loaded onto a 5-ml HisTrap FF column. The column flow-through was collected and dialyzed against Tris-HCl buffer (0.1 M, pH 8.0 at 4 °C) containing NaCl (0.5 M) and 10% (v/v) glycerol. Tag-less LrPduO used for kinetic analysis was stored at −80 °C until used. In Vitro Adenosyltransferase Activity Assay—Activity assays were performed as described (37) with the following modifications. The final volume of each reaction was 1 ml. Empty, sealed quartz cuvettes were flushed with oxygen-free N2 for 5 min. Under a stream of O2-free N2, Tris-HCl (0.2 M; pH 8.0 at 37 °C),

![FIGURE 1. LrPduO compensates for the lack of CobA enzyme. All strains used in these studies carried a null allele of the mefE gene (encodes the B12-independent methionine synthase) and a deletion of the cobA gene in their chromosome. Cells were grown in NCE minimal medium as described under “Experimental Procedures.” The growth conditions used for this analysis demanded adenosylation of the precursor cobinamide prior to its conversion to AdoCbl. The following growth rates (ΔOD650 h−1) were calculated for each strain using the GraphPad Prism® v4 software package: cobA+/cobA− (closed circles), 0.15 ± 0.11; cobA/pET-15b (open triangles, empty vector control), 0.001; cobA/ pSecobA+ (open circles, positive control), 0.11 ± 0.006; cobA/pLrpduO+ (closed triangles, experiment), 0.105 ± 0.009.](image-url)
hydroxocobalamin (HOCbl 0.1–10 \( \mu \)M), MgCl\(_2\) (0.5 mM), ATP (1 \( \mu \)M-1 mM), and Ti(III)citrate (0.1 mM) were added to the cuvette in the order stated. After all additions were made, flushing was continued for 30 s. To ensure that all Co(III) was reduced to Co(I), reaction mixtures were incubated at 37 °C for 5 min. The adenosylation reaction was initiated by the addition of \( L_r \)PduO protein (45 nM). Adenosylcobalamin (AdoCbl) formation was monitored at 388 nm.

Crystallization and Data Collection—Crystals of the recombinant native protein, expressed with a 35-amino acid, N-terminal-(His)\(_6\) tag, were grown at 20 °C in hanging drop by mixing 4 \( \mu \)l of 20 mg/ml protein in buffer C with 4 \( \mu \)l of precipitant solution composed of HEPPS (0.1M, pH 8.5), ammonium sulfate (1.9 M, pH 8.5), MgCl\(_2\) (0.1 M), and HOCbl (4 mM). The resulting solution was microseeded after 24 h, resulting in the growth of cubic-shaped, pink crystals (\( a = b = c = 110.6 \) Å). Crystals were transferred to a synthetic mother liquor solution containing HEPPS, (0.11M, pH 8.5), ammonium sulfate (1.1M, pH 8.5), ATP (2 mM), MgCl\(_2\) (55 mM), NaCl (165 mM), and HOCbl (15 mM) and allowed to soak for 7 days. The coloration of the crystals darkened from pink to red over the course of the soak. The soaked crystals were transferred in five steps to a cryoprotectant solution consisting of HEPPS (0.11 M, pH 8.5), ammonium sulfate (1.2 M, pH 8.5), ATP (3 mM), MgCl\(_2\) (55 mM), NaCl (0.3 M), HOCbl (15 mM), and glycero1 (20% v/v) and were flash cooled in a nitrogen stream at 100 K (Oxford Cryosystems, Oxford, UK). The crystals belonged to the space group I23, with one subunit in the asymmetric unit and the unit cell parameters

Structure Determination and Refinement—The structure was determined by molecular replacement with the program MOLREP (39) starting from the model for TA1434, a PduO-type cob(I)alamin adenosyltransferase from \( T. \) acidi\( p \)holium (PDB accession identifier 1NOG, Ref. 23), for which there is 31% identity with the protein sequence of \( L_r \)PduO. Residues 3–181 for the \( L_r \)reuteri enzyme were built automatically into the electron density by the program ARP/WARP (40), and the structure was refined with the program REFMAC (41). The initial structure was subject to manual verification. Multiple conformations were added with the program COOT (42). Water molecules were added to the dataset by ARP/WARP with subsequent manual verification. The final model, refined to 1.68-Å resolution, includes residues Lys\(^7\)–Asn\(^{182}\) of \( L_r \)PduO and the heteroatom Mg-ATP with the following 15 residues displaying multiple conformations: Gln\(^{19}\), Lys\(^{45}\), Ile\(^{48}\), Val\(^{54}\), Ser\(^{56}\), Glu\(^{60}\), Phe\(^{67}\), Ser\(^{83}\), Gln\(^{90}\), Val\(^{105}\), Ala\(^{108}\), Arg\(^{128}\), Arg\(^{132}\), Gln\(^{140}\), Met\(^{178}\). Residues Met\(^1\) and Ser\(^{183}\)–Arg\(^{188}\), and those residues corresponding to the preceding 35 amino acids of the N-terminal tag were disordered and were not built into the model. The side chains corresponding to Arg\(^{14}\), Glu\(^{66}\), Lys\(^{85}\), Gln\(^{110}\), Lys\(^{111}\), Gln\(^{146}\), Arg\(^{91}\), Asn\(^{182}\) were disordered and could not be built into the electron density map. Despite the mild red coloration of crystals grown and soaked in the presence of HOCbl, no electron density was observed in the map that could be attributed to fully occupied HOCbl. All figures of molecular structures were generated with the program PyMOL. A Ramachandran plot shows that 96.9% of the residues are in the most favored region, with one outlier that lies in a flexible loop. Refinement statistics are summarized in Table 2.
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TABLE 3  
Kinetic parameters for native and N-terminally tagged LrPduO

| Substrate varied | N-terminally tagged LrPduO | Untagged LrPduO (NH₂-GSASPMLV₂K₃) |
|------------------|---------------------------|-----------------------------------|
|                  | $K_m$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1} \mu$M$^{-1}$) | $K_m$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1} \mu$M$^{-1}$) |
| HOCh₁ | 0.31 ± 0.05 | 2.8 ± 0.2 × 10⁻² | 9.0 ± 1.6 × 10⁴ | 0.13 ± 0.01 | 2.4 ± 0.1 × 10⁻² | 1.8 ± 0.2 × 10⁵ |
| ATP | 3.3 ± 0.8 | 2.8 ± 0.1 × 10⁻² | 8.5 ± 2.1 × 10³ | 2.2 ± 0.1 | 2.6 ± 0.1 × 10⁻² | 1.2 ± 0.1 × 10⁴ |

FIGURE 3. Ribbon representations of ATP-bound LrPduO. a, stereoview of a single subunit of LrPduO colored in rainbow format, beginning with blue at the N terminus and ending in red at the C terminus. A stick representation showing the position of Mg-ATP at the N-terminal binding site is also labeled. b, stereoview of the trimeric enzyme with the 3-fold axis of symmetry indicated. A space-filling representation denotes the positions of Mg-ATP.

RESULTS AND DISCUSSION

The LrPduO Protein Has ATP:Co(I)rrinoid Adenosyltransferase Enzyme Activity—Results from complementation studies involving LrPduO protein are shown in Fig. 1. The growth defect of a strain of S. enterica lacking the housekeeping ATP:co(I)rrinoid adenosyltransferase (CobA) enzyme was corrected by the presence of a plasmid encoding the LrPduO protein (Fig. 1, closed versus open triangles). Under the growth conditions used in this study, S. enterica does not express endogenous PduO, nor does it express eutT (44, 45). Therefore, the conditions of the above-mentioned experiments demanded that the LrPduO protein adenosylate cobinamide prior to its conversion to AdoCbl (46). Hence, on the basis of these data, it is concluded that LrPduO is involved in corrinoid adenosylation. Given the homology of LrPduO to the N-terminal domain of S. enterica PduO (SePduO; Fig. 2), it was hypothesized that the LrPduO protein catalyzes the formation of the unique Co-C bond between the 5’-deoxyadenosyl moiety of ATP and the cobalt ion of the corrin ring.

Kinetics of the Reaction Catalyzed by LrPduO—Direct evidence that LrPduO catalyzes the last step of the corrinoid adenosylation pathway (13) was obtained in vitro. Initial velocity kinetic determinations were performed using chemically reduced cob(I)alamin and ATP as substrates for purified LrPduO protein that did or did not have an N-terminal tag. Kinetic constants were measured by holding one substrate at saturation while the other was varied (Table 3). These data confirmed that the LrPduO protein has ATP:cob(I)alamin adenosyltransferase enzyme activity. The apparent $K_m$ for ATP ($K_m$, 2 μM) at saturating cob(I)alamin is similar to the one reported for the human PduO-type adenosyltransferase ($K_m$, 6 μM) (20, 47), but is lower than that reported for the PduO enzymes from T. acidophilum ($K_m$, 110 μM) (23) and S. enterica ($K_m$, 20 μM) (48). Notably, the apparent $K_m$ for cob(I)alamin at saturating ATP is at least 10-fold lower than any previously reported $K_m$ value for any type of ATP:adenosyltransferase ($K_m$, 1–5 μM). The apparent $k_{cat}$ for the L. reuteri enzyme (0.03 s$^{-1}$) is also lower than what has been reported for other adenosyltransferases ($k_{cat} = 0.1–0.3$ s$^{-1}$), bringing the $k_{cat}/K_m$ values for the L. reuteri enzyme within the range of what has been reported for the other enzymes ($k_{cat}/K_m = 10⁸–10⁹$ M$^{-1}$ s$^{-1}$). Our results indicate...
that the presence of the 35-residue N-terminal tag has only a small effect on the reaction kinetics. Similar kinetic results have been reported for the N-terminal GST-tagged constructs of hATR (47) and the mitochondrial targeting sequence-tagged bovine PduO (19). Based on the location of the active site at the immediate N terminus of LrPduO (see the structural description below), the slightly elevated $K_m$ values for the tagged enzyme may reflect a minor impedance of access to the active site with a concomitant reduction in the overall binding affinity.

**Overall Structure and Description of the Active Site**—The structure of LrPduO was determined for the N-terminally tagged protein using molecular replacement, with initial phases from the crystal structure of TA1434 (PDB accession identifier 1NOG), the putative PduO from M. tuberculosis (2G2D) aligned with the $\alpha$-carbon backbone of LrPduO (shown in red) using the program ALIGN (55). The r.m.s. deviations for alignments excluding the N-terminal 23 residues of LrPduO were 0.63, 1.10, and 1.26 Å with the enzymes from B. subtilis, T. acidophilum, and M. tuberculosis, respectively.

The neighboring subunit. Unlike the inverted P-loop observed for CobA (12), the ATP binding site of LrPduO does not conform to any of the classic nucleotide binding structural motifs as seen in the ATP grasp or protein kinase families. The N-terminal ATP-binding loop is made up of several highly conserved residues and is clamped into position through a salt bridge with the positions and orientations of the phosphates and the nucleotide ring very well defined (Fig. 5a). The N terminus accounts for the lower half of the enzyme active site with additional residues being contributed from helix four and five of the structure of LrPduO (shown in red) and the putative PduO from M. tuberculosis (2G2D) aligned with the $\alpha$-carbon backbone of LrPduO (shown in red) using the program ALIGN (55). The r.m.s. deviations for alignments excluding the N-terminal 23 residues of LrPduO were 0.63, 1.10, and 1.26 Å with the enzymes from B. subtilis, T. acidophilum, and M. tuberculosis, respectively.

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FIGURE 4. Structural overlay of PduO-type adenosyltransferase enzymes. Stereoview of the $\alpha$-carbon backbone of B. subtilis YveK (1RTY), T. acidophilum PduO (1NOG), and the putative PduO from M. tuberculosis (2G2D) aligned with the $\alpha$-carbon backbone of LrPduO (shown in red) using the program ALIGN (55). The r.m.s. deviations for alignments excluding the N-terminal 23 residues of LrPduO were 0.63, 1.10, and 1.26 Å with the enzymes from B. subtilis, T. acidophilum, and M. tuberculosis, respectively.

structure of LrPduO is unique in that it includes an additional ordered 23 amino acids at the immediate N terminus of the protein (Fig. 4). The newly defined structure of the N terminus spans residues 2–24 of the native polypeptide chain and does not include any contributions from the 35-residue N-terminal tag. Aside from this ordered N-terminal loop, no significant conformational changes accompany ATP binding, with low r.m.s. deviations (0.6–1.3 Å) for the superposition of the LrPduO structure with three structures of the apoenzyme. The N terminus is well-ordered (average B-value, 23.4) and consists of a region of random coil with a helical twist followed by two antiparallel $\beta$-strands interspaced by a loop of five residues. Electron density is clearly observable for Mg-ATP within this N-terminal cleft with the positions and orientations of the phosphates and the nucleotide ring very well defined (Fig. 5a). The N terminus accounts for the lower half of the enzyme active site with additional residues being contributed from helix four and five of the overall backbone structure of this loop is kept rigid through a pair of backbone hydrogen bonds (Lys$^9$–Asp$^5$; Gly$^8$–Gly$^{11}$) that preserve a tight helical twist leading into the first $\beta$-strand. Both Gly$^9$ and Gly$^{11}$ are conserved among homologs of SePduO, further suggesting a key role in scaffolding the structure of the ATP binding loop. It appears that MgATP binding is responsible for ordering the N-terminal loop. Unfortunately, despite co-crystallization with HOCbl, only small lobes of difference electron density were observed in the vicinity of the active site, indicating low occupancy for this ligand. The binding region for HOCbl, therefore, could not be modeled with confidence. However, identification of the binding site for MgATP does place restraints on the binding site for HOCbl.

**ATP Binding at the Active Site of LrPduO**—Mg-ATP is bound through direct interactions with 9 residues contributed from adjacent subunits of the trimer (Fig. 5, b and c). A total of 6 residues whose side chains are directly involved in binding are conserved among PduO-type adenosyltransferase enzymes (Fig. 2). This includes the hydroxyl of Thr$^{13}$, which appears to be structurally conserved as either a serine or threonine. The
majority of enzyme-substrate interactions are centered on the triphosphate of ATP. As such, these interactions are expected to contribute significantly to ordering the N-terminal loop upon ATP binding. Several of these interactions originate from main chain amide hydrogens and carbonyls on the N-terminal loop, lending importance to the conservation of Gly_8 and Gly_11 in maintaining the overall fold. The phosphate oxygen atoms of the enzyme-bound ATP are positioned to provide 3 coordination bonds to Mg^{2+}. In addition, two highly ordered water molecules along with the carbonyl oxygen of Asn_156 provide the remaining coordination to the bipyramidal Mg^{2+}. Whereas many interactions with the triphosphate of ATP originate from a conserved sequence motif at the N terminus, this sequence motif appears to be unique to this class of enzymes, despite its high sequence conservation among the PduO-type adenosyltransferases (Fig. 2). A search of the protein database using the conserved sequence Thr-(Lys/Arg)-X-Gly-Asp-X-Gly-X-(Thr/Ser), corresponding to residues 5–13 of the LrPduO phosphate binding domain, revealed no significant similarities with existing protein classes. Whereas the conserved amino acid sequence of PduO loosely shares features of the consensus triphosphate binding sequence of the mechanistically similar adenosylmethionine synthetase and inorganic pyrophosphatase (49), a structural comparison with the active site of adenosylmethionine synthetase (50) reveals no striking similarities in the overall fold responsible for ATP binding. PduO catalyzes the elimination of triphosphate from ATP (14, 48), a relatively uncommon mechanism among ATP-utilizing enzymes.

The conserved residue Arg_132 is poised to play a critical role in stabilizing developing negative charge in the transition state and also in properly orienting the C5'-carbon of ATP for nucleophilic attack through two hydrogen bonding interactions.
contacts with the bridging oxygen of ribose (2.7 Å) and the bridging oxygen to the α-phosphate (2.4 Å). Whereas the precise nature of its catalytic role awaits detailed mutagenic analysis, it is noteworthy that an Arg to His mutation of the structurally equivalent residue in hATR results in a complete loss of enzyme activity (47). Interestingly, CobA has no comparable protein-ligand contacts centered around the C-5’ carbon, reinforcing the notion that the PduO- and CobA-type adenosyltransferase enzymes are mechanistically distinct despite their common overall reactions and similar efficiencies. Arg132, along with the neighboring residue, Arg138, displays alternate conformations in the crystal structure. In the absence of HOCbl it is difficult to assess which set of conformations is mechanistically important.

In addition to the contact between the Arg132 side chain and the bridging ribose, Glu135 and the main chain carbonyl oxygens of Arg132 and Arg14 are the only apparent bonding interactions between the protein and the adenosine base. Of these, only the side chain of Glu135 contacts the C-6 amine that is specific to adenine. This small number of contacts may facilitate the transfer of adenosine to cobalamin and the subsequent leaving of the first product, AdoCbl. This lack of a stringent set of binding contacts combined with a lack of tight packing around the base moiety also suggests an active site with sufficient conformational freedom to accommodate nucleotides other than ATP. While CobA type adenosyltransferases have long been known to accommodate alternate nucleotides (10, 14, 25, 51), the TaPduO and SePduO accept only ATP (23, 48). However, the human PduO enzyme does display moderate catalytic activity with several alternate nucleotides (19).

A survey of LrPduO relative specific activities for a series of alternate nucleotides revealed specificity for a broad set of nucleotides (Table 4) with relative efficiencies similar to those described for hATR. While a detailed structural interpretation of the relative observed rates requires further kinetic analysis, it is interesting to note the low specific activity of ITP compared with ATP. The difference between these two nucleotides is limited to the identity of the functional group at the C-6 position of the purine base. Substitution at C-6 of the hydrogen bond-donating amine of ATP for the hydrogen bond-accepting carbonyl of ITP results in a 100-fold reduction in specific activity. Provided that this reduction in activity results primarily from a loss of binding affinity, the reduced specific activity with ITP may be attributed to the loss of a critical hydrogen bonding interaction with the backbone carbonyl of Arg132. GTP, which also has a carbonyl at C-6, may recover a portion of this lost binding energy through a favorable potential hydrogen bonding interaction between its C-2 amine and the backbone carbonyl of Val258. It is, however, more difficult to structurally interpret the reduced specific activities observed for CTP and UTP on account of the smaller relative size of the purine bases.

A subset of PduO-type enzymes is now emerging that can be defined by the ability of the enzyme to accept alternate nucleotides in place of ATP. In addition, the $K_m$ of LrPduO for ATP (3 μM) is closer to that reported for the human enzyme (7 μM) than it is to those values reported for the SePduO (18 μM) or TaPduO (110 μM) enzymes. Interestingly, neither humans nor lactobacilli have an identified CobA homolog, leaving the PduO-type adenosyltransferase as the only enzyme available for the conversion of Cbl to cobenzyme B$_{12}$. In contrast, the genome of _T. acidophilum_ does contain a gene encoding a putative CobA homolog (30% identical to SeCobA). Whether the presence of a CobA-type enzyme has an effect on the specificity and efficiency of PduO enzymes remains an open question.

### Table 4

| Nucleotide substrate | Relative activity |
|----------------------|------------------|
| ATP                  | 100 ± 2%         |
| 2′-deoxy-ATP         | 13 ± 1%          |
| ADP                  | 6.6 ± 0.4%       |
| AMP                  | None detected    |
| GTP                  | 36 ± 4%          |
| CTP                  | 1.4 ± 0.1%       |
| ITP                  | 0.8 ± 0.2%       |
| UTP                  | None detected    |

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The crystal structure of LrPduO, like that of CobA (12), reveals ATP bound in a deep cleft at the active site. In CobA, the C-5’ carbon of ribose is positioned such that it is poised for nucleophilic attack by the reduced cobalt atom bound above. A similar scenario would be expected for the binding of cob(1)alamin in PduO. However, despite the high apparent binding affinity of cob(1)alamin for the enzyme at saturating concentrations of ATP, attempts to co-crystallize the enzyme in the presence of 2 mM HOCbl and to soak the crystals in 15 mM HOCbl did not result in any interpretable electron density for this substrate in the final structure. The crystals gained a mild red coloration when co-crystallized with HOCbl and subsequently gained a deeper red coloration upon soaking, indicating some degree of binding. Whereas HOCbl is expected to have a lower affinity than reduced cob(1)alamin, its dissociation constant for the human enzyme has been measured fluorometrically as ~9 μM (52). It is likely that the high concentration of ammonium sulfate in the crystallization solutions significantly reduces the overall binding affinity of HOCbl for the enzyme. Attempts to co-crystallize the TaPduO enzyme with cobalamin have also been unsuccessful (23). Nevertheless, it is likely that the corrinoid substrate is bound immediately adjacent to ATP in a large surface-exposed hole (Fig. 6). PduO catalyzes the direct transfer of the adenosyl moiety from ATP to reduced cobalamin, without proceeding through an enzyme-bound adenosylated intermediate (48). For such a direct transfer to take place, the corrinoid must be bound in close proximity to ATP. The hole immediately adjacent to ATP is large enough to accommodate the corrinoid but the orientation of the tetrapyrole ring cannot be predicted because side chains lining the pocket will adopt different conformations on binding. Indeed, some lobes of electron density were interspersed throughout this hole at a signal level significantly exceeding background noise ($σ > 3$) and several residues lining this cavity (Asn19, Phe67, Ser83, Ala108, Arg128, Arg132) display alternate conformations. Furthermore, this cavity encompasses a localized area of positive electrostatic potential which may serve either to assist in binding the corrinoid substrate negatively charged phosphate moiety and/or to promote docking of
Active Site of PduO Adenosyltransferase

the putative reductase that is responsible for reducing bound cob(I)alamin to cob(II)alamin (20).

Mechanistic Insights into Methylmalonic Aciduria—The kinetic and structural characterization of the PduO-type enzyme from L. reuteri provides insights into the recent description of mutations in hATR responsible for methylmalonic aciduria (23, 47, 53, 54). Even though the sequence similarity between the human enzyme and LrPduO enzyme is only 39%, the similar kinetic constants and nucleotide specificity provide a structural framework for understanding the biochemical consequences of the mutations. Importantly, the majority of disease-related mutations are clustered around residues contributed from helix 5 to the active site (Fig. 7). Interestingly, no mutations have been identified within the phosphate binding region on the N-terminal loop.

The R186W mutation of hATR is particularly common in patients, accounting for ~30% of sequenced alleles and is associated with early onset of methylmalonic aciduria (54). Purification and characterization of the mutant enzymes R186W and R186A in vitro has been shown to result in a complete loss of activity (23, 47). The structurally equivalent residue of the LrPduO enzyme is Arg128. Whereas this residue does not directly interact with ATP, it is expected to play a critical role in catalysis as evidenced by its absolute conservation among PduO-type adenosyltransferases and the complete loss of enzyme activity that results from mutations at this position. There are several possible roles for Arg128. 1) Its location in the corrinoid binding pocket results in an important specific interaction with the corrin ring. Efforts to co-crystallize LrPduO with various corrinoid substrates are continuing to better define the interactions between the enzyme and the corrinoid substrate. 2) It interacts directly to properly position or stabilize Arg132 for catalysis, or 3) it forms a critical subunit-subunit contact through a salt bridge with the absolutely conserved Asp35 on the adjacent subunit. On this note, it is interesting that in hATR the R186W mutation results in apparently unstable protein in vivo (47), suggesting that a stabilizing subunit-subunit contact is lost in these mutant enzymes.

Both R190C and R190H have been identified in patients with methylmalonic aciduria (54). This residue is structurally equivalent to Arg132 in the LrPduO enzyme and is expected to be essential to catalysis as discussed above. It is also now clear that the E193K mutation found in patients with methylmalonic aciduria (equivalent to Glu135 in LrPduO) will result in the loss of a specific interaction with ATP in the active site and in the loss of a critical secondary residue responsible for positioning one of two Mg2+-coordinating water molecules in the active site.

The structure of LrPduO presented here answers many of the questions of how nucleotides bind to this class of adenosyltransferase, but other questions remain. In particular, knowledge of how the corrinoid binds in the active site is required to establish a molecular mechanism for adenosyltransfer and the
role of conserved residues. The current study establishes the foundation for these future investigations.

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