# Purification and Initial Biochemical Characterization of ATP:Cob(I)alamin Adenosyltransferase (EutT) Enzyme of *Salmonella enterica*

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# Purification and Initial Biochemical Characterization of ATP:Cob(I)alamin Adenosyltransferase (EutT) Enzyme of *Salmonella enterica*[*] [S]

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**ATP:cob(I)alamin adenosyltransferase (EutT) of *Salmonella enterica* was overproduced and enriched to ~70% homogeneity, and its basic kinetic parameters were determined. Abundant amounts of EutT protein were produced, but all of it remained insoluble. Soluble active EutT protein (~70% homogeneous) was obtained after treatment with detergent. Under conditions in which cobalamin (Cbl) was saturating, $K_{m(ATP)}$ = 10 μM, $k_{cat}$ = 0.03 $s^{-1}$, and $V_{max}$ = 54.5 nM $min^{-1}$. Similarly, under conditions in which MgATP was saturating, $K_{m(Cbl)}$ = 4.1 μM, $k_{cat}$ = 0.06 $s^{-1}$, and $V_{max}$ = 105 nM $min^{-1}$. Unlike other ATP:co(I)rrinoid adenosyltransferases in the cell (*i.e.* CobA and PduO), EutT activity was ≥50-fold higher with ATP *versus* GTP, and EutT retained 80% of its activity with ADP substituted for ATP and was completely inactive with AMP as substrate, indicating that the enzyme requires the β-phosphate group of the nucleotide substrate. The data suggest that the amino group of adenine might play a role in nucleotide recognition and/or binding. Unlike the housekeeping CobA enzyme, EutT was not inhibited by inorganic tripolyphosphate ($PPP_i$). Results from $^{31}P$ NMR spectroscopy studies identified $PP_i$ and $P_i$ as by-products of the EutT reaction. In the absence of Cbl, EutT cleaved ATP into adenosine and $PPP_i$, suggesting that $PPP_i$ is broken down into $PP_i$ and $P_i$. Electron transfer protein partners for EutT were not encoded by the *eut* operon. EutT-dependent activity was detected in cell-free extracts of *cobA* strains enriched for EutT when FMN and NADH were used to reduce cob(III)alamin to cob(I)alamin.**

ATP:co(I)rrinoid adenosyltransferases are enzymes responsible for the conversion of vitamin $B_{12}$ (cobalamin (Cbl)[3]) into coenzyme $B_{12}$ (adenosyl-$B_{12}$, adenosylcobalamin (AdoCbl)) (Fig. 1). In the enterobacterium *Salmonella enterica* serovar Typhimurium LT2 (hereafter *S. enterica*), there are three different classes of ATP:co(I)rrinoid adenosyltransferases. The housekeeping ATP:co(I)rrinoid adenosyltransferase of this bacterium is encoded by the *cobA* gene, which is constitutively expressed (1). Two other ATP:co(I)rrinoid adenosyltransferases (PduO and EutT) (2–4) are encoded by large operons, the functions of which are needed for the catabolism of 1,2-propanediol (the *pdu* (propanediol utilization) operon) (5) or ethanolamine (the *eut* (ethanolamine utilization) operon) (6, 7, 17).

Bioinformatics analysis of the CobA, PduO, and EutT proteins shows that they evolved from different ancestors. The best characterized of the three enzymes, CobA adenosylates incomplete and complete corrinoids; its three-dimensional structure complexed with its substrates has been solved; and its physiological electron donor partner (*i.e.* flavodoxin A) is known (8–13). The PduO enzyme has been studied in some detail, and knowledge of how it works is of particular interest because of its homology to ATP:co(I)rrinoid adenosyltransferases found in mammals, including humans (14, 15). Structure studies of the archaeal PduO homolog of *Thermoplasma acidophilum* have provided insights into the basis for methylmalonic aciduria in humans (16). The EutT enzyme is the least understood of all three types of ATP:co(I)rrinoid adenosyltransferases. Identification of *eutT* as the gene encoding the ATP:co(I)-rrinoid adenosyltransferase of the operon was reported recently (3, 4). The reason for the diversity in the lineage of these proteins is not known.

There are many open questions regarding PduO and EutT function. As mentioned above, we do not know the identity of the electron donor that generates the cob(I)alamin nucleophile needed for formation of the Co–C bond, nor do we understand how PduO and EutT bind ATP or their corrinoid substrates. To better understand the molecular details of how the EutT enzyme works, we purified the protein and began the biochemical characterization of its enzyme activity.

## EXPERIMENTAL PROCEDURES

### *Assessment of Growth Phenotypes*

Strains were grown overnight in 2 ml of lysogenic broth (19, 20) containing the appropriate antibiotic; cultures were incubated at 37 °C. Growth behavior was analyzed using 96-well plates containing 190 μl of no-carbon E medium (21), 50 mM ethanolamine (pH 7), trace minerals elixir (22), 1 mM $MgSO_4$, 50 mM $NH_4Cl$), 0.5 mM L-methionine, and 200 nM hydroxocobalamin (HOCbl) and inoculated with a 10-μl sample (~$2 \times 10^7$ colony-forming units) of an overnight lysogenic broth culture. Plates were placed in the chamber of a BioTek plate reader with the temperature held at 37 °C; shaking was constant at a maximum setting.

### *Plasmid Construction*

The strains, plasmids, and primers used in this study are listed in Tables 1 and 2.

*Plasmid pEUT12*—The *S. enterica eutT*$^+$ gene was amplified by PCR using the appropriate primers. The NdeI/XhoI fragment containing the native stop codon was cloned into pTYB2 (New England Biolabs Inc.) to yield plasmid pEUT12 for overproduction of wild-type EutT in *Escherichia coli*.

*Plasmids pEUT25–27*—Plasmids pEUT25–27 encode EutT variants that were constructed using the QuikChange site-directed mutagenesis

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1s.

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[3] The abbreviations used are: Cbl, cobalamin; AdoCbl, adenosylcobalamin; HOCbl, hydroxocobalamin; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; $PPP_i$, inorganic tripolyphosphate.

kit (Stratagene). Plasmid pEUT12 was used as template. Mutations in plasmids pEUT25–27 were verified by DNA sequencing.

*Plasmids pEUT35–37*—To avoid indirect effects by unknown mutations introduced into the cloning vector, the mutant alleles were moved from pEUT25–27 into a non-mutagenized cloning vector and placed under the control of a tunable promoter. For this purpose, primers shown in supplemental Table 1s (see plasmids pEUT35–37) were used to amplify the mutant *eutT* alleles from their respective pYTB2 plasmids (pEUT25–27). Amplification products were cut with EcoRI and XbaI restriction enzymes and placed under the control of the arabinose-inducible $P_{araBAD}$ promoter in plasmid pBAD24 (23) cut with the same enzymes. The resulting plasmids were named pEUT36 (*eutT1168*, EutT(C79A)), pEUT37 (*eutT1169*, EutT(C80A)), and pEUT38 (*eutT1170*, EutT(C83A)). These plasmids were used in *in vivo* studies aimed at determining the functionality of the proteins encoded by the mutant *eutT* alleles. For this purpose, plasmids were transformed (24) into strain JE7180 (*metE205 ara-9 cobA366*::Tn*10*d(*cat*$^+$) *eutE18*::MudI1734(*kan*$^+$)). Growth of the resulting strains was assessed as described above.

*Plasmids pEUT55–58*—Wild-type chromosomal alleles of *eutP*, *eutQ*, *eutT*, and combinations of them were constructed by PCR amplification using the primers shown in supplemental Table 1s. Amplification products were cloned under the control of the $P_{araBAD}$ promoter in vector pBAD24 using the appropriate restriction sites.

*Plasmid pFDX1*—The *S. enterica* ferredoxin gene (*fdx*$^+$) was amplified, and the PCR fragment was cut with NdeI and BamHI restriction enzymes and cloned into plasmid pET-16b (Novagen), yielding plasmid pFDX1, which encodes ferredoxin with a hexahistidine tag fused to its N terminus.

*DNA Sequencing*—All plasmids were sequenced using BigDye® protocols (ABI PRISM) at the DNA Sequencing Facility of the Biotech Center at the University of Wisconsin (Madison, WI).

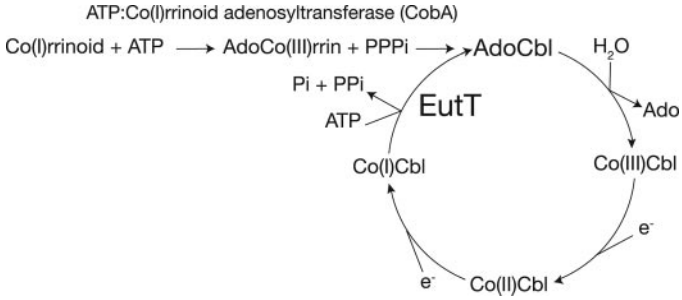


FIGURE 1. **Contributions of the CobA and EutT ATP:co(I)rrinoid adenosyltransferases to the pool of AdoCbl in *S. enterica*.**

### *Enrichment of EutT Protein*

A transformant of *E. coli* BL21(λDE3) carrying the appropriate *eutT*$^+$ plasmid was used to inoculate two sterile culture tubes (16 × 150 mm) containing 5 ml of lysogenic broth plus ampicillin (100 μg/ml) and grown overnight at 37 °C. The next morning, the 10-ml starter culture was added to 500 ml of lysogenic broth plus ampicillin, trace minerals, cysteine (0.5 mM), and methionine (0.5 mM) in a 2-liter Erlenmeyer flask. The culture was shaken at 140 rpm for 1.5 h at 37 °C. Isopropyl β-D-thiogalactopyranoside was added to 0.5 mM, and the temperature was dropped to 15 °C for overnight incubation. Cells were harvested by centrifugation at 7354 × *g* in a Beckman Coulter Avanti J-25I centrifuge fitted with an LA-16.250 rotor at 4 °C for 10 min. Cell paste was transferred to a 50-ml serum vial, flushed with $O_2$-free $N_2$ on ice for 10 min, and frozen at −80 °C under 100 kilopascals of pressure until used.

Cells were broken under anoxic conditions. For this purpose, frozen cell paste was transferred to an anoxic chamber and resuspended in 10 ml of anoxic lysis buffer (1× BugBuster™ reagent (Novagen), 20 mM glycine, and 20 mM CHES buffer (pH 9.5) containing 1 mM phenylmethylsulfonyl fluoride). The cell suspension was incubated at room temperature for 20 min with intermittent agitation (approximately every 5 min). The lysate was moved into a stainless steel centrifuge tube fitted

**TABLE 1**

**Strains and plasmids used in this study**

Unless indicated otherwise, all strains and plasmids were constructed during the course of this work. WT, wild-type.

| Strain | Genotype | Source |
|---|---|---|
| ***E. coli* BL21(λDE3)** | F$^-$ *dcm ompT hsdS*($r_B^-$ $m_B^-$) *gal* λ(DE3) | New England Biolabs |
| ***S. enterica* TR6583** | *metE205 ara-9* | J. R. Roth via K. Sanderson |
| **Derivatives of TR6583** | | |
| JE1096 | *cobA343*::MudI1734(*kan*$^+$)[a] | Lab collection |
| JE1293 | *cobA366*::Tn*10*d(*cat*$^+$) | Lab collection |
| JE8292 | *cobA343*::MudI1734(*kan*$^+$) *zfa3648**Tn*10**zfa3649*(Δ*eut*) | |
| JE7172 | *cobA366*::Tn*10*d(*cat*$^+$)/pEUT7 | |
| JE7179 | *cobA366*::Tn*10*d(*cat*$^+$) *eutE18*::MudI1734(*kan*$^+$) | Lab collection |
| JE7180 | *cobA366*::Tn*10*d(*cat*$^+$) *eut1141*(Δ*eutT*)[b] | Lab collection |
| JE8815 | *eutP1168*(Δ*eutP*)[b] | |
| JE8816 | *eutQ1169*(Δ*eutQ*)[b] | |
| JE8817 | *eutPQ1170*(Δ*eutPQ*)[b] | |
| JE8818 | *cobA343*::MudI1734(*kan*$^+$) *eutP1168*(Δ*eutP*) | |
| JE8819 | *cobA343*::MudI1734(*kan*$^+$) *eutQ1168*(Δ*eutQ*) | |
| JE8820 | *cobA343*::MudI1734(*kan*$^+$) *eutPQ1169*(Δ*eutPQ*) | |

| Plasmid | Genotype | Protein | Ref./Source |
|---|---|---|---|
| pEUT7 | *bla*$^+$ $P_{araBAD}$-*eutT*$^+$ | WT EutT | 3 |
| pEUT36 | *bla*$^+$ *eutT1171* | EutT(C79A) | |
| pEUT37 | *bla*$^+$ *eutT1172* | EutT(C80A) | |
| pEUT38 | *bla*$^+$ *eutT1173* | EutT(C83A) | |
| pEUT55 | *bla*$^+$ $P_{araBAD}$-*eutP*$^+$ | WT EutP | |
| pEUT56 | *bla*$^+$ $P_{araBAD}$-*eutQ*$^+$ | WT EutQ | |
| pEUT57 | *bla*$^+$ $P_{araBAD}$-*eutPQ*$^+$ | WT EutPQ | |
| pEUT58 | *bla*$^+$ $P_{araBAD}$-*eutPQT*$^+$ | WT EutPQT | |
| pFPR1 | *bla*$^+$ *fpr*$^+$ | WT Fpr | Lab collection |
| pFLDA4 | *bla*$^+$ *fldA*$^+$ | FldA-$His_6$ | 3 |
| pFDX1 | *bla*$^+$ *fdx*$^+$ | $His_6$-Fdx | |

[a] Referred to as MudJ (32).
[b] In-frame deletion constructed using described methodology (33).

**TABLE 2**
**ATP:Cob(I)alamin adenosyltransferase assays with mutant EutT proteins**
WT, wild-type.

| Fraction | Specific activity[a] | WT |
|---|---|---|
| | | % |
| **WT** | 61 ± 0.36 | 100 |
| CHAPS-soluble | | |
| **EutT(C79A)** | | |
| Whole lysate | 1.4 ± 0.25 | 2 |
| CHAPS-soluble | 12 ± 1.5 | 20 |
| Insoluble[b] | 14 ± 0.49 | 23 |
| **EutT(C80A)** | | |
| Whole lysate | 0.04 ± 0.06 | <0.1 |
| CHAPS-soluble | 0.37 ± 0.11 | 0.6 |
| Insoluble[b] | 0.71 ± 0.61 | 1 |
| **EutT(C83A)** | | |
| Whole lysate | 0.37 ± 0.02 | 0.6 |
| CHAPS-soluble | 0.75 ± 0.02 | 1 |
| Insoluble[b] | 0.28 ± 0.04 | 0.5 |

[a] The average of three determinations using Ti(III) citrate as reductant.
[b] Measured after solubilization with CHAPS.

with an expandable O-ring cap and centrifuged at 43,667 × *g* for 30 min at 4 °C in a Beckman Coulter Avanti J-25I centrifuge fitted with a JA-25.50 rotor. The centrifuged lysate was moved back to the anoxic chamber, where the supernatant was decanted. Pelleted cell membranes and insoluble material were resuspended in 10 ml of anoxic solution containing 8 mM CHAPS in 20 mM glycine and 20 mM CHES buffer (pH 9.5). Repeated pipetting ensured homogeneous resuspension of the pellet, which was then incubated at room temperature for 1 h with gentle agitation every 10 min. The suspension was centrifuged at 43,667 × *g* for 1 h at 4 °C in stainless steel tubes fitted with sealable caps. The tubes were transferred back into the anoxic chamber, where the soluble material was decanted; 200-μl samples were dispensed into glass serum vials fitted with gray butyl rubber stoppers; and aluminum seals were crimped in place. Sealed vials were removed from the chamber, pressurized with $N_2$ (100 kilopascals), and kept at 4 °C until used.

### *Protein Purity Analysis*

Protein purity was assessed by SDS-PAGE (25) using the FOTO/Eclipse® electronic documentation and analysis system (FOTODYNE Inc.), including the software packages FOTO/Analyst® PC Image (Version 5.0) and TotalLab™ (Version 2003; Nonlinear Dynamics Ltd.) for one-dimensional gel analysis.

### *Overexpression and Isolation of Fpr, FldA, and Fdx*

Fpr and FldA proteins were overproduced and isolated as reported previously (12, 26). Fresh *E. coli* BL21(λDE3) (JE3892)/pFDX1 transformants were grown in two 10-ml lysogenic broth cultures containing ampicillin (100 μg/ml) and incubated overnight at 37 °C with shaking. Each 10-ml starter culture was used to inoculate 2 liters of Terrific Broth supplemented with 0.1 mg/ml ferrous ammonium sulfate and 100 μg/ml ampicillin (27); inoculated flasks were incubated at 37 °C for 15 h. Cells were harvested by centrifugation at 5000 × *g* at 4 °C in a Beckman Coulter Avanti J-20XPI centrifuge fitted with a JLA-8.1000 rotor, and the cell paste was stored at −80 °C until used. The cell paste was resuspended in 20 mM potassium $P_i$ buffer (pH 7.4) containing 0.5 M NaCl and 1 mM phenylmethylsulfonyl fluoride, passed through a French pressure cell twice at 1250 kilopascals, and treated with DNase for 10 min on ice. Cell-free extracts were centrifuged at 43,000 × *g* for 30 min at 4 °C in a Beckman Coulter Avanti J-25I centrifuge fitted with a JA-25.50 rotor and then passed through a 0.2-μm syringe filter to remove residual particulate material. N-terminally $His_6$-tagged ferredoxin protein was purified on an ΔKTA FPLC Explorer using a 5-ml HiTrap chelating HP column (Amersham Biosciences) charged with $NiSO_4$. Fractions containing pure protein were pooled, concentrated in an Amicon Centricon YM10 unit (cutoff of 10 kDa), dialyzed against 20 mM potassium $P_i$ buffer (pH 7.4) containing 0.2 M NaCl, and concentrated again in an Amicon Microcon YM10 unit. Total yield was ~1 mg of EutT protein/10 g of cell paste.

### *Preparation of Cell-free Extracts*

*S. enterica* strains were grown in no-carbon E medium containing trace minerals elixir, $MgSO_4$, $NH_4Cl$, and methionine at concentrations indicated above. In addition, 1 μM HOCbl (or AdoCbl as indicated), 200 μM L-(+)-arabinose as inducer, and 50 mM ethanolamine (pH 7) or 30 mM glycerol were added. Cells in stationary phase were harvested by centrifugation at 4 °C, transferred to a 50-ml serum vial, and flushed with anoxic $N_2$ gas. Cells were broken at room temperature after resuspension in anoxic lysis buffer. The cell suspension was kept at room temperature for 30 min with intermittent agitation. The protein concentration was determined using the Bradford reagent (Bio-Rad) according to the manufacturer's instructions. Cell-free extracts were used immediately for corrinoid adenosylation assays.

### *Corrinoid Adenosylation Assays*

Quartz cuvettes fitted with removable silicon rubber septa (Starna Cells, Inc.) were flushed with $O_2$-free $N_2$ and filled with degassed buffer containing 0.2 M Tris-Cl buffer (pH 7) at 37 °C. The concentration of HOCbl ranged between 0.5 and 50 μM; $MnCl_2$ was present at 0.5 mM. Ti(III) citrate (2.2 mM) was used to reduce the corrin from $Co^{3+}$ to $Co^{+}$; ATP was added (0.5 μM to 1 mM); and 0.5 μM purified CHAPS-EutT protein or 50 μg of cell-free extract was used per reaction. CHAPS-EutT protein was warmed to room temperature prior to its addition to the reaction mixture. A temperature shift to 37 °C was used to start the reaction, the rate of which was monitored by the decrease in the abundance of cob(I)alamin substrate at 388 nm. When FldA or Fdx was used as the reductant, Ti(III) citrate was replaced with 2 μM Fpr protein, 0.5 mM NADPH, and 1 μM purified N-terminally $His_6$-tagged ferredoxin or C-terminally $His_6$-tagged flavodoxin A protein. In assays in which cell-free extract was used in lieu of purified CHAPS-EutT protein, 50 μM FMN, 1 mM NADH, and 1 mM ATP were used in place of Ti(III) citrate. Corrinoid adenosylation was assessed by the change in absorbance at 525 nm of the sample after 30 min of incubation at 37 °C and after photolysis for 10 min on ice.

### *$^{31}$P NMR Spectroscopy Studies*

A 10-ml corrinoid adenosylation reaction mixture containing 3 mM Ti(III) citrate, 100 μM ATP, 100 μM HOCbl, 0.5 mM $MgCl_2$, and 16 μM EutT in 0.2 M Tris-Cl buffer (pH 7) at 37 °C was incubated for 2 h at 37 °C. After incubation, EDTA was added to 20 mM; reactions were concentrated overnight at room temperature under vacuum to 0.5 ml in an SPD111V SpeedVac® concentrator (Thermo Savant); and 100% $D_2O$ was added to a final concentration of 6% (v/v). Standards were added to 100 μM as indicated. $^{31}$P NMR spectra were acquired at the Nuclear Magnetic Resonance Facility of the University of Wisconsin (Madison).

### *Chemicals*

All chemicals were obtained from Sigma unless indicated otherwise and were of high purity. Ti(III) citrate was prepared from liquid $TiCl_3$. Briefly, 0.2 M citrate was adjusted to pH 8. The citrate solution was degassed with $O_2$-free $N_2$ in a sealed glass serum vial for 20 min. $TiCl_3$ was added with a glass syringe to the serum vial with a pressurized $O_2$-free $N_2$ headspace. The final Ti(III) citrate concentration was 88 mM.

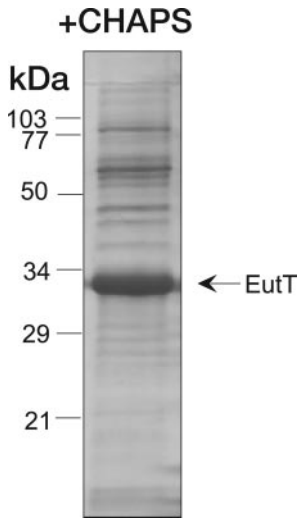


FIGURE 2. **Expression and isolation of the wild-type EutT protein.** Proteins from cell fractions were separated by SDS-PAGE (25). Protein mass markers are shown on the *left*.

## RESULTS

### *Initial Purification and Biochemical Characterization of EutT Protein*

Recombinant EutT protein was overproduced in *E. coli*, but remained insoluble unless cells containing EutT protein were lysed under anoxic conditions using CHES/glycine buffer (pH 9.5), followed by resuspension of EutT from the pelleted material with 8 mM CHAPS and a final centrifugation step. After these purification steps, EutT was substantially enriched (~70% homogeneous) (Fig. 2). However, EutT could not be resolved from contaminating proteins by column chromatography. All biochemical assays were performed using CHAPS-solubilized EutT.

### *EutT Activity as a Function of Reductant and pH*

EutT activity were erratic when $KBH_4$ was used as reductant to convert cob(III)alamin to cob(I)alamin *in vitro*. To circumvent this problem, Ti(III) citrate was used as reductant in all Cbl adenosylation assays. The highest EutT activity was measured at pH 7 (specific activity of 34 nmol of AdoCbl $min^{-1}$ $mg^{-1}$ of protein) and was <30% active at higher pH (*e.g.* at pH 7.5, specific activity of 9.7 nmol of AdoCbl $min^{-1}$ $mg^{-1}$; at pH 8, specific activity of 7.3; and at pH 8.5, specific activity of 7.3).

### *Metal Requirement and Inhibition by $Ni^{2+}$*

Several metals were tested for their ability to stimulate EutT activity (*i.e.* $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Na^+$, and $K^+$). Only $Mn^{2+}$ ions stimulated EutT enzyme activity (by 32%) above the no-metal control (Fig. 3*A*). Surprisingly, $Ni^{2+}$ and $Zn^{2+}$ ions inhibited EutT activity by 62 and 77%, respectively. Fifty percent inhibition was the maximum level measured when the ratio of $Ni^{2+}$ ion to EutT monomer was 100:1 (at 100 $\mu$M $Ni^{2+}$)] (Fig. 3*B*). The shape of the inhibition curve suggested single-site binding.

### *Cysteinyl Residues in the Cys-rich Motif of EutT Are Required for Activity*

EutT has a putative $HX_7HX_3CCX_2C$ motif that resembles the cytochrome oxidase $CCX_2C$ dicopper-binding site or a 4Fe/4S cluster that was recently identified in the *Bacillus megaterium* CbiX protein (Fig. 4) (28). This cysteine-rich motif of EutT was targeted for site-directed mutagenesis to determine whether $Cys^{79}$, $Cys^{80}$, and $Cys^{83}$ are important for structural integrity, enzyme catalysis, or both. Residues were mutated to Ala, and the resulting enzymes were tested for their ability to function *in vivo* and *in vitro*. None of the alleles coding for EutT(C79A), EutT(C80A), or EutT(C83A) complemented the *S. enterica cobA eutT* strain (JE7180) during growth on ethanolamine (data not shown). Variant proteins were overproduced and purified using the protocol used for wild-type EutT protein, and their adenosyltransferase activity was assayed *in vitro* (Table 2). EutT(C79A) was the most active of the three variants tested, with 20% of the wild-type activity detected in the detergent-soluble fraction. In contrast, EutT(C80A) and EutT(C83A) retained very little activity (0.6 and 1.2%, respectively). Mutant EutT proteins were found predominantly in the detergent-insoluble pellet, likely misfolded and aggregated (Table 2). We propose that $Cys^{79}$ might be more important for structural integrity than $Cys^{80}$ and $Cys^{83}$.

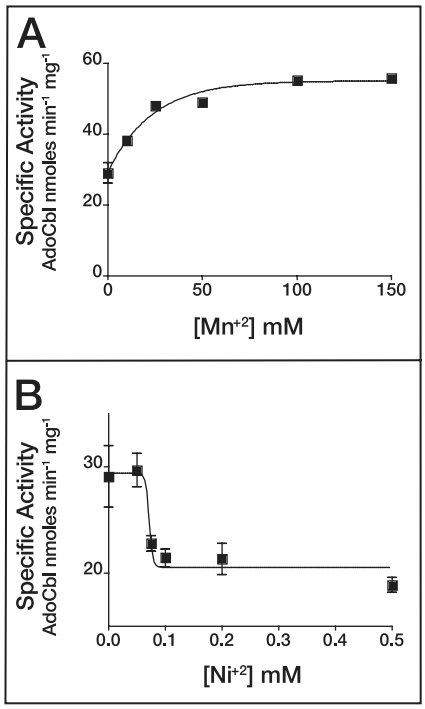


FIGURE 3. **Metal requirements and $Ni^{2+}$ inhibition of EutT adenosyltransferase activity.** *A*, enhancement of Cbl adenosyltransferase activity as a function of added $Mn^{2+}$ ions; *B*, inhibition of EutT activity by $Ni^{2+}$ ions. The shape of the curve suggests competition for a single site.


FIGURE 4. **Comparison of the cysteine-rich motif of EutT and the 4Fe/4S cluster motif of *B. subtilis* CbiX.** EutT has three cysteines (boldface) and two histidines (*outlined*) that seem to be reminiscent of the $CCX_2C$ dicopper-binding motif of cytochrome oxidase and/or the $MXCX_2C$ 4Fe/4S motif of *B. subtilis* CbiX. $Cys^{79}$, $Cys^{80}$, and $Cys^{83}$ of EutT were changed to alanine to assess their importance in EutT function.

### *Substrate Specificity*

*Nucleotide Substrate*—Under conditions in which Cbl was saturating, the $K_m$ and $k_{cat}$ for ATP were measured at 10 $\mu$M and 0.03 $s^{-1}$, respectively (Fig. 5*A*); the $V_{max}$ was calculated at 54.5 nM $min^{-1}$. Order-of-addition experiments revealed a modest increase in the specific activity of the enzyme (17%) when it was incubated with ATP first (specific activity of 42 *versus* 36 nmol of AdoCbl $min^{-1}$ $mg^{-1}$ of protein). Recognition of NTPs by EutT was unlike that by CobA or PduO (2, 10). Not surprisingly, ATP was the preferred nucleotide substrate (Fig. 6*A*), but there were several differences between CobA and EutT in terms of the nucleotide substrate each enzyme could use. EutT retained reasonable activity (31%) when dATP was the substrate, whereas CobA did not use dATP as substrate at all. EutT retained 38% activity when CTP was used

as substrate, whereas CobA used CTP 1.5-fold better than ATP. EutT activity was poor (<10%) with GTP, UTP, and ITP, whereas CobA used them 45, 125, and 85% as effectively as ATP (10). The sharpest difference between CobA and EutT was the ability of the latter to use ADP as substrate, retaining 83% of its activity relative to ATP. CobA did use ADP as substrate.

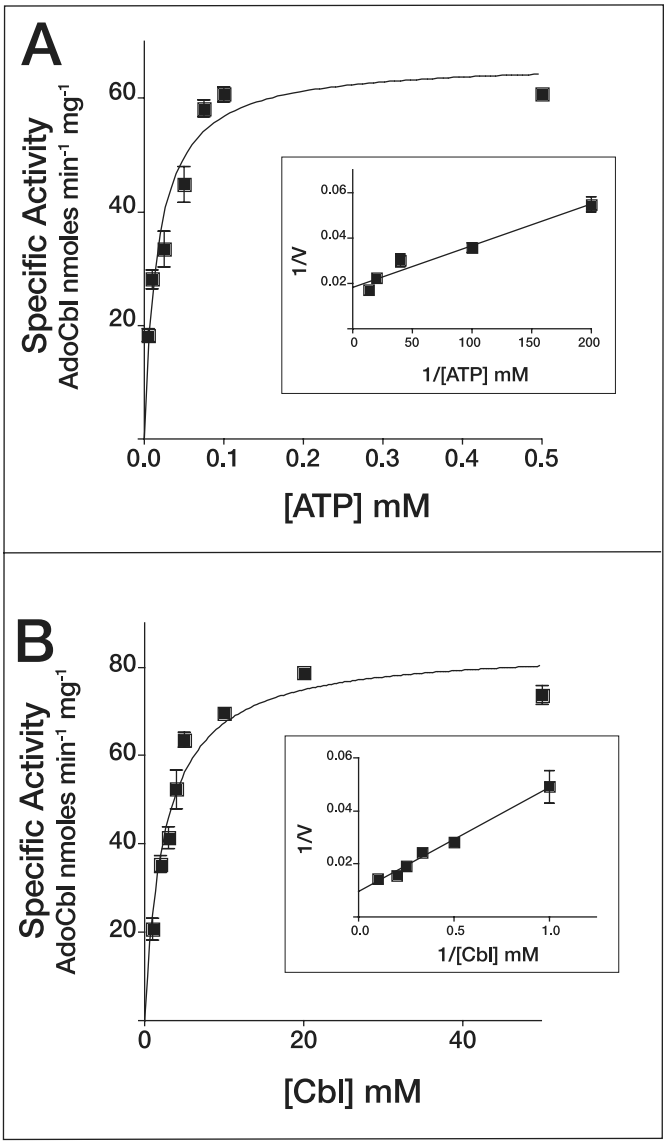


FIGURE 5. **Kinetics of ATP and Cbl binding to EutT.** *A*, Cbl was held at 50 $\mu$M, whereas the ATP concentration was varied. $K_{m(ATP)}$ = 10 $\mu$M, $k_{cat}$ = 0.03 $s^{-1}$, and $V_{max}$ = 54 nM $min^{-1}$. *B*, ATP was held at 500 $\mu$M, whereas the Cbl concentration was varied. $K_{m(Cbl)}$ = 4.1 $\mu$M, $k_{cat}$ = 0.06 $s^{-1}$, and $V_{max}$ = 105 nM $min^{-1}$. The *insets* show Lineweaver-Burk plots.

*Corrinoid Substrate*—We also determined the kinetic parameters of EutT for Cbl under conditions in which MgATP was saturating. Under such conditions, $K_m$ = 4.1 $\mu$M, $k_{cat}$ = 0.06 $s^{-1}$, and $V_{max}$ = 105 nM $min^{-1}$ (Fig. 5*B*). *In vitro*, EutT adenosylated cobinamide, an incomplete corrinoid lacking the 5,6-dimethylbenzimidazole lower ligand. When cobinamide was used as substrate, the specific activity of the enzyme (13 nmol of adenosylcobinamide $min^{-1}$ $mg^{-1}$ of protein) was 21% of the that measured when Cbl was the substrate (specific activity of 62 nmol of AdoCbl $min^{-1}$ $mg^{-1}$ of protein), suggesting a role for the nucleotide loop in substrate binding. Despite its *in vitro* activity, a mutant *cobA* strain (JE7172) (Table 1) expressing the *eutT*$^+$ allele from a plasmid failed to grow on ethanolamine or glycerol as sole carbon source when cobinamide was provided in the medium. Clearly, the rate of cobinamide adenosylation measured *in vitro* was not enough to satisfy even the lowest cellular requirement for Cbl, *i.e.* the level needed for methylation of homocysteine to methionine by Cbl-dependent methionine synthase (MetH).

*EutT Cleaves Inorganic Tripolyphosphate (PPP$_i$) into PP$_i$ and P$_i$*—To date, corrinoid adenosyltransferases (10, 29) and *S*-adenosylmethionine synthase (30) are the only enzymes known to release $PPP_i$ as a reaction by-product without cleaving it to $PP_i$ and $P_i$. CobA is strongly inhibited by $PPP_i$ (10), and PduO is not (2). EutT was slightly inhibited by $PPP_i$ (15%) and $PP_i$ (20%) when each compound was present at 10-fold the concentration of ATP in the reaction mixture. $P_i$ did not inhibit EutT activity (Fig. 6*B*). Because $PPP_i$ only slightly inhibited EutT, we investigated whether EutT cleaves $PPP_i$. For this purpose, we used $^{31}P$ NMR spectroscopy to determine whether $PPP_i$, $PP_i$, and $P_i$ are stable reaction by-products. NMR data showed that, whereas ATP and $PPP_i$ were present in the complete reaction (Fig. 7*B*), $PP_i$ and $P_i$ peaks were enhanced. When Cbl was omitted from the reaction mixture, only $PPP_i$ signals were observed (Fig. 7*A*). Therefore, unlike CobA and PduO, EutT cleaved $PPP_i$ to $PP_i$ and $P_i$ and cleaved ATP to adenosine and $PPP_i$ in the absence of Cbl.

### *Insights into the Identity of the Electron Transfer Protein Partner for EutT in Vivo*

*Candidates within the eut Operon*—We investigated the possibility that a protein encoded by the *eut* operon might be the electron transfer protein for EutT. We focused on two genes of unknown function, *eutP* and *eutQ*. Chromosomal in-frame deletions of these genes were constructed singly or in combination. In a mutant *cobA* background, each of these deletions was tested for its ability to affect EutT-dependent growth on ethanolamine. If *eutP*, *eutQ*, or both are required

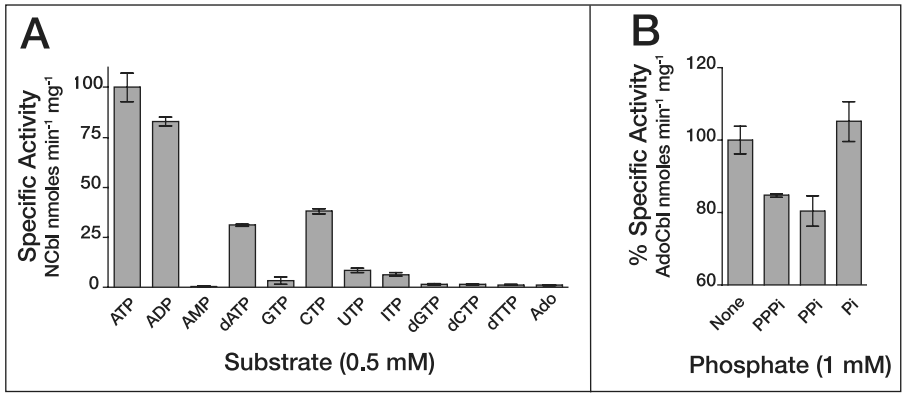


FIGURE 6. **NTP substrate analogs and phosphate product inhibition of EutT.** *A*, NTPs (0.5 mM) were tested in lieu of ATP. *B*, $PPP_i$, $PP_i$, or $P_i$ was added to 10-fold the amount of ATP present in the reaction to assess product inhibition. For ATP, the specific activity was 61 nmol $min^{-1}$ $mg^{-1}$.

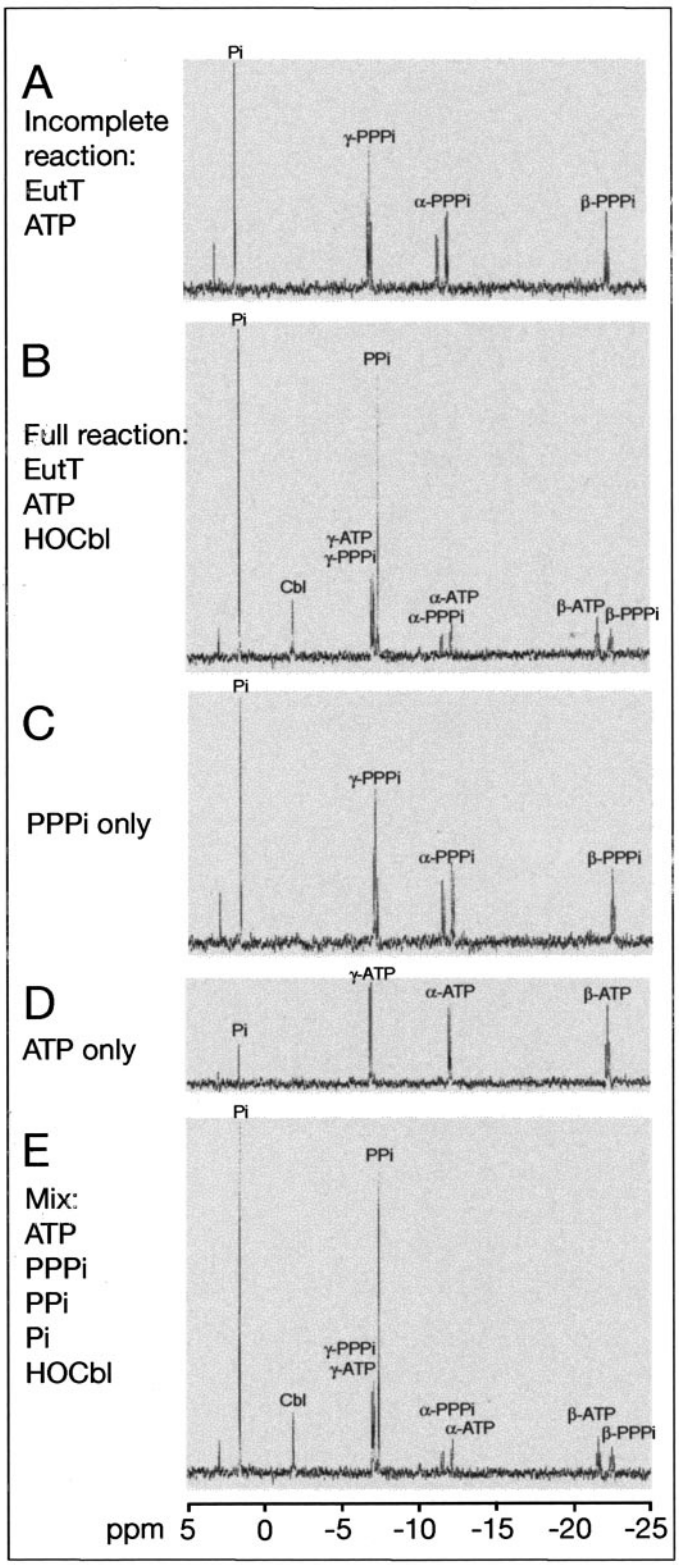


FIGURE 7. **$^{31}$P NMR spectra of EutT reaction products.** *A*, reaction mixture lacking Cbl; *B*, complete reaction mixture containing EutT, ATP, and Cbl; *C* and *D*, $PPP_i$ and ATP standards, respectively; *E*, mixture of ATP, $PPP_i$, $PP_i$, $P_i$, and HOCbl standards without EutT.

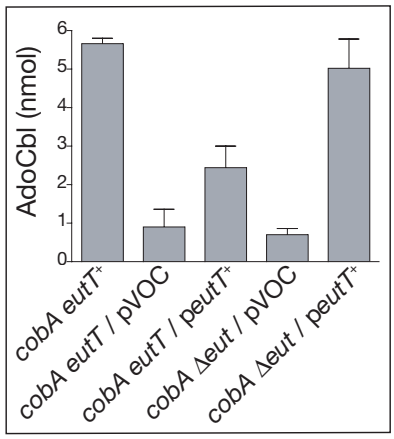


FIGURE 8. **NADH/FMN-dependent adenosylation of Cbl by EutT.** Cell-free extracts of several strains of *S. enterica* were prepared, and 50 $\mu$g of total protein was used in adenosylation assays. The product was detected by change in the absorbance at 525 nm after photolysis. *pVOC*, vector-only control; *peutT*$^+$, pEUT7. Several culture media were tested. Cell-free extracts of strains used in these studies were as follows (from left to right): strain JE1293 (*cobA eutT*$^+$) grown in no-carbon E medium containing ethanolamine (30 mM) and HOCbl (1 mM); strains JE7204 (*cobA eutT*/vector-only control) and JE7205 (*cobA eutT*/pEUT7) grown in no-carbon E medium containing ethanolamine (30 mM), AdoCbl (1 mM), and L(+)-arabinose (200 mM); and strains JE8941 (*cobA* Δ*eut*/vector-only control) and JE8942 (*cobA* Δ*eut*/pEUT7) grown in no-carbon E medium containing glycerol (30 mM), l(+)-arabinose (200 mM), and methionine (0.5 mM). The high level of HOCbl or AdoCbl was used to allow faster growth and higher yield. In all cases, reaction mixtures contained FMN (50 $\mu$M), NADH (1 mM), and ATP (1 mM).

for cob(II)alamin → cob(I)alamin reduction, mutant strains lacking these functions will be unable to grow on ethanolamine as carbon and energy source, *i.e.* the phenotype will be identical to that of the *cobA eutT* strain (JE7180). If EutP and/or EutQ protein is not required for other metabolic functions, lack of these functions will not affect growth on ethanolamine when the wild-type *cobA* or *eutT* gene is present on the chromosome. The *cobA eutQ* strain (JE8819) displayed a slightly reduced growth rate on ethanolamine relative to the *cobA eutQ*$^+$ strain (JE1293) (generation time of 0.26 *versus* 0.23 doublings/h). However, this minor effect of the *eutQ* mutation on ethanolamine metabolism was independent of Cbl adenosylation because the *eutQ cobA*$^+$ strain (JE8816) also had a slightly reduced growth rate compared with the wild-type strain (TR6583) (generation time of 0.24 *versus* 0.29 doublings/h). The *cobA eutP* strain (JE8818) grew as well as the *cobA* strain JE1293 (data not shown). None of the *eutQ*, *eutP*, or *eutPQ* mutant strains were defective for growth on ethanolamine as sole carbon and energy source, as reported for the *cobA eutT* strain (JE7180) (3). Based on the above data, we concluded that neither EutP nor EutQ is required for EutT function.

*Candidates outside the eut Operon*—Given the highly hydrophobic nature of EutT protein, we postulated that a plausible source of electrons for EutT function could be the electron transport system. Various electron donors were tested for the ability to allow AdoCbl production as a function of the presence of unresolved lysate, cob(III)alamin, ATP, and $Mn^{2+}$. Lysate from cells expressing *eutT* from an overexpression vector supported the conversion of cob(III)alamin to AdoCbl when FMN and NADH were included in the reaction mixture (Fig. 8). Control experiments showed that FMN or NADH alone did not result in EutT-dependent AdoCbl synthesis (data not shown). EutT-dependent activity was detected regardless of the presence of the *eut* operon on the *S. enterica* chromosome, indicating that the EutT function does not require interactions with other Eut proteins.

AdoCbl was not synthesized from cob(III)alamin when purified CHAPS-EutT protein was substituted for EutT-enriched cell-free extracts not treated with CHAPS under conditions requiring FMN and NADH (Fig. 8). AdoCbl was not detected when the membrane fraction of the *cobA eutT* strain (JE7180) was added to purified CHAPS-EutT protein, suggesting that, in the presence of detergent, factors that allow EutT function no longer interact with EutT.

The electron transfer proteins flavodoxin (FldA) and the 2Fe/2S ferredoxin (Fdx) were tested for their ability to reduce cob(II)alamin in the active site of purified CHAPS-EutT enzyme. *S. enterica* ferredoxin (flavodoxin)-NADP(H) reductase (Fpr) was used to reduce FldA and Fdx. Although activity was detectable, it was <1% of the activity measured when Ti(III) citrate was used to reduce EutT (specific activity of 0.49 *versus* 61 nmol of AdoCbl $min^{-1}$ $mg^{-1}$ of protein). At present, we cannot rule out the participation of FldA or Fdx in EutT function.

## DISCUSSION

The three classes of ATP:co(I)rrinoid adenosyltransferases known to date, CobA, PduO, and EutT, are not evolutionarily related as shown by their profound differences at the amino acid sequence level. The biochemical characterization of these enzymes has provided basic knowledge regarding substrate binding and mechanism of catalysis.

*ATP Binding and By-products of EutT Are Different from Those of Other Adenosyltransferases*—From the work reported here, we have learned that the EutT enzyme requires the amino group of adenine and the β-phosphate for ATP binding and that the enzyme cleaves ATP to $PPP_i$ and adenosine when Cbl is absent from the active site. Unlike CobA or PduO, EutT cleaves $PPP_i$ to $PP_i$ and $P_i$, explaining why $PPP_i$ is a poor inhibitor of its adenosyltransferase activity. The elucidation of three-dimensional EutT structures could provide further mechanistic insights into substrate binding.

*Is EutT Located outside or inside the Metabolosome?*—Although the data strongly suggest that EutT is associated with the cell membrane, the data available do not distinguish whether EutT is inside or outside the metabolosome. One possibility is that EutT is part of a complex involving other Eut proteins, that the complex is housed within the metabolosome, and that EutT anchors the latter to the cell membrane. Alternatively, EutT may be associated with the cell membrane outside of the metabolosome and may not interact with other Eut proteins at all. The latter possibility is feasible because the metabolosome is not a sealed proteinaceous vessel (31), and AdoCbl could diffuse into the metabolosome for use by ethanolamine ammonia-lyase. Some bacteria with ethanolamine ammonia-lyase genes (*eutBC*) do not have a *eutT* homolog in their chromosome and presumably rely solely on the housekeeping CobA enzyme. It is unknown whether these organisms metabolize ethanolamine as sole carbon and energy source as do *S. enterica* and *E. coli*, both of which maintain *eutT* within the *eut* operon.

*Possible Means for the Generation of the Cob(I)alamin Nucleophile for the EutT Reaction*—The data obtained in these studies shed some light on the identity of the electron donor of EutT. It is unclear whether ferredoxin (Fdx) or flavodoxin A (FldA) is the physiological electron transfer protein partner of EutT. The poor activity of EutT when Fdx or FldA was used to reduce cob(II)alamin to cob(I)alamin may reflect limited productive interactions between Fdx or FldA and EutT because of the detergent needed to solubilize EutT. The fact that some activity was detected with FldA and Fdx warrants further investigation. On the other had, the phenotypic behavior of strains JE8818 (*cobA eutP*), JE8819 (*cobA eutQ*), and JE8820 (*cobA eutPQ*) strongly suggests that the EutP and EutQ proteins are not involved in EutT-dependent corrinoid adenosylation or in cob(II)alamin → cob(I)alamin reduction. The function of the EutP and EutQ proteins remains unknown.

A membrane-soluble electron donor may provide the reducing power for the reduction of the corrinoid substrate of EutT. We note that EutT-dependent adenosylation of cob(III)alamin by cell-free extracts enriched for EutT was achieved in the presence of NADH and FMN. One plausible explanation for these results is that NADH sinks its electrons into the electron transport system, and a semiquinone from dihydroflavins ($FMNH_2$ and $FADH_2$) or dihydroquinone reduces cob(II)alamin to cob(I)alamin in the active site of EutT. There is a need, however, to explain why NADH and FMN did not support EutT-dependent corrinoid adenosylation when the reaction mixture contained purified EutT in lieu of EutT-enriched cell-free extracts. In thinking about this problem, one must consider that purified EutT was complexed with a detergent (CHAPS). The fact that EutT adenosylated chemically reduced cob(I)alamin, but could not accept electrons from the NADH/FMN pair, suggests that CHAPS does not affect EutT activity as long as the co(I)rrinoid substrate is available.

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