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

Nicole R. Buan§ and Jorge Escalante-Semerena*
Department of Bacteriology, University of Wisconsin-Madison
Running title: the EutT corrinoid adenosyltyrantransferase of Salmonella
*Corresponding author: 1710 University Av., Madison, WI 53726-4087
Telephone: 608-262-7379; FAX: 608-265-7909; E-mail: escalante@bact.wisc.edu

The ATP:Co(I)balamin adenosyltransferase (EutT) enzyme of Salmonella enterica was overproduced, 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 where Cbl was saturating, the $K_m^{ATP} = 10 \mu M$, $k_{cat} = 0.03 s^{-1}$, and $V_{max} = 54.5$ nM min$^{-1}$. Similarly, under conditions where MgATP was saturating, $K_m^{Cbl} = 4.1 \mu M$, $k_{cat} = 0.06 s^{-1}$, and $V_{max} = 105$ nM min$^{-1}$. Unlike other ATP:corrinoid adenosyltransferases in the cell (i.e. CobA, PduO), EutT activity was >50-fold higher with ATP vs GTP, retained 80% of its activity when ADP substituted for ATP, and was completely inactive with AMP as substrate, indicating that the enzyme required the $\beta$-phosphate group of the nucleotide substrate. The data suggested 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 PPI. Results from $31P$-NMR spectroscopy studies identified PP$_i$ and P$_i$ as by-products of the EutT reaction. In the absence of cobalamin, EutT cleaved ATP into adenosine and PPP$_i$, suggesting that PPP$_i$ was 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.

EXPERIMENTAL PROCEDURES

Microbiological Techniques

Assessment of growth phenotypes. Strains were grown overnight in 2 ml of lysogenic broth (LB)
(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 (NCE) medium (21), ethanolamine (50 mM, pH 7), trace minerals elixir (22), MgSO₄ (1 mM), NH₄Cl (50 mM), L-methionine (0.5 mM), hydroxocobalamin (200 nM, HOCl), and inoculated with a 10-µl sample (~2x10⁷ cfu) of an overnight LB culture. Plates were placed in the chamber of a BioTek plate reader whose temperature was held at 37°C; shaking was constant at maximum setting.

**Recombinant DNA Techniques**

**Plasmid construction.** Strains, plasmids and primers used in this study are listed in Tables 1,2.

Plasmid pEUT12. The *S. enterica* eutT gene was amplified by PCR using the appropriate primers. The *NdeI*/Xhol fragment containing the native stop codon was cloned into pTyb2 (New England Biolabs) to yield plasmid pEUT12 for overproduction of EutT_WT in *E. coli*.

Plasmid pEUT25-27. Plasmid pEUT25-27 encode EutT variants which were constructed using the Site-Directed Mutagenesis™ 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 were placed under the control of a tunable promoter. For this purpose, primers shown in Table 1s (supplemental online data; see plasmids pEUT35-37) were used to amplify the mutant alleles from their respective pTyb2 plasmids (pEUT25-27). Amplification products were cloned under the control of the ParaBAD promoter in vector pBAD24 using the appropriate restriction sites.

Plasmid pFDX1. The *S. enterica* ferredoxin gene (fdx*) was amplified, the PCR fragment was cut with *NdeI* and BamHI restriction enzymes and cloned into plasmid pET-16b (Novagen) yielding plasmid pFDX1, which encoded ferredoxin with a hexahistidine tag fused to its N terminus. DNA sequencing. All plasmids were sequenced using BigDye® protocols (ABI PRISM) by the DNA sequencing Facility at the Biotech Center at the University of Wisconsin-Madison.

**Biochemical Techniques**

**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 mm x 150 mm) containing 5 ml of LB plus ampicillin (100 µg/ml) and grown overnight at 37°C. The next morning, the 10 ml starter was added to 500 ml LB ampicillin, trace minerals, cysteine (0.5 mM), methionine (0.5 mM) in a 2-liter Erlenmeyer flask. The culture was shaken at 140 rpm at 37°C for 1.5 h. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.5 mM and the temperature was dropped to 15°C for overnight incubation. Cells were harvested by centrifugation at 7,354 x g at 4°C for 1 h. The cell paste was transferred to a 50-ml serum vial, flushed with O₂-free N₂ on ice for 10 min and frozen at -80°C under 100 kPa of pressure until use.

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 1X Bug Buster™ reagent (Novagen) in 20 mM glycine / 20 mM N-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer pH 9.5 containing 1 mM phenylmethylsulfonylfluoride (PMSF). The cell suspension was incubated at room temperature for 20 min with intermittent agitation (approx. every 5 min). The lysate was moved into a stainless-steel centrifuge tube fitted with an expandable O-ring cap, centrifuged at 4°C for 30 min at 43,667 x g in a Beckman Coulter Avanti J-
protein was purified on a ÄKTA FPLC Explorer resuspended in 20 mM KPi buffer pH 7.4 stored at -80°C until used. Cell paste was harvested by centrifugation at 4°C at 5,000 x g. Cell-free extracts were passed through a French press cell twice at 1250 kPa and containing 0.5 M NaCl, 1 mM PMSF, passed through a 0.2 µM syringe filter to remove residual particulate material. H6Fdx protein was purified on a ÄKTA FPLC Explorer using a 5-ml HiTrap Chelating HP column (Amersham) charged with NiSO₄. Fractions containing pure protein were pooled, concentrated in an Amicon YM10 Centricon unit (cut off = 10 kDa), dialyzed against 20 mM KP₃ buffer pH 7.4 containing 0.2 M NaCl, and concentrated again in an Amicon YM10 Microcon unit. Total yield was approximately 1 mg of EutT protein per 10 g of cell paste.

**Preparation of cell-free extracts.** *S. enterica* strains were grown in NCE minimal medium containing trace minerals elixir, MgSO₄, NH₄Cl, methionine at concentrations described above. In addition, 1 µM HOCl or (AdoCbl as indicated), 200 µM L(+)-arabinose as inducer, and ethanolamine (50 mM, pH 7) or glycerol (30 mM) were added. Cells in stationary phase were harvested by centrifugation at 4°C, transferred to a 50-ml serum vial and flushed with anoxic N₂ gas. Cells were broken at room temperature after resuspension in anoxic lysis buffer [Bug Buster reagent (Novagen), 20 mM glycine / 20 mM CHES buffer pH 9.5, containing 1 mM PMSF]. The cell suspension was kept at room temperature for 30 min with intermittent agitation. Protein concentration was determined using the Bradford Reagent (Bio-Rad Laboratories) according to manufacturer’s instructions. Cell-free extracts were used immediately for corrinoid adenosylation assays.

**Corrinoid adenosylation assays.** Quartz cuvettes fitted with removable silicon rubber septa (Starina Cells) were flushed with O₂-free N₂ and filled with degassed buffer containing 0.2 M TrisCl buffer (pH 7, 37°C). HOCl was added between 0.5-50 µM MnCl₂ was added. Ti(III)citrate (2.2 mM) was used to reduce the corrin from Co³⁺ to Co¹⁺, ATP was added (0.5 µM-1mM), and 0.5 µM HOCl (or AdoCbl as indicated), purified CHAPS/EutT protein or 50 µg 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 whose rate was monitored by the decrease in the abundance of cob(Ⅰ)alamin substrate at 388 nm. When FldA or Fdx were used as reductant, Ti(III)citrate was replaced by 2 µM Fpr protein, 0.5 mM NADPH and 1 µM of purified H6Fdx (N-terminal H₆ tag) or FldAH₆ (C-terminal H₆ tag) proteins. In assays where 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.

31P-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 MgCl₂, 16 µM EutT in 0.2 M TrisCl buffer (pH 7, 37°C) was incubated for 2 h at 37°C. After incubation, ethylenediaminetetraacetic acid (EDTA) was added to 20 mM, reactions were concentrated under vacuum to 0.5 ml in a SPD111V SpeedVac® (Thermo Savant) concentrator overnight at room temperature; 100% D₂O was added to a final concentration of 6% (v/v). Standards were added to 100 µM when indicated. 31P-NMR spectra were acquired at the Nuclear Magnetic Resonance Facility at Madison (NMRFAM) at the University of Wisconsin-Madison.

Chemicals. All chemicals were obtained from Sigma unless otherwise indicated, and were of high purity. Ti(III)citrate was prepared from liquid TiCl₃. Sigma unless otherwise indicated, and were of high purity. Ti(III)citrate was prepared from liquid TiCl₃.

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 detergent, and a final centrifugation step. After these purifications 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 KBH₄ was used as a 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 cobalamin adenosylation assays. The highest EutT activity was measured at pH 7 (sp. act. 34 nmol of AdoCbl min⁻¹ mg⁻¹ of protein) and less than 30% active at higher pH (e.g. at pH 7.5, sp. act = 9.7; at pH 8, sp. act = 7.3; at pH 8.5, sp. act = 7.3 nmol AdoCbl min⁻¹ mg⁻¹).

Metal requirement and inhibition by Ni²⁺. Several metals were tested for their ability to stimulate activity of EutT (i.e. Ca²⁺, Mg²⁺, Zn²⁺, Na⁺, K⁺). Only Mn²⁺ ions stimulated EutT enzyme activity (by 32%) above the no-metal-added control (Fig. 3A). Surprisingly, Ni²⁺ and Zn²⁺ ions inhibited EutT activity (62% and 77% activity, respectively. Fifty percent inhibition was the maximal level measured when the ratio of Ni²⁺ ion:EutT monomer was 100:1 [at 100 µM Ni²⁺] (Fig. 3B). The shape of the inhibition curve suggested single-site binding.

Cysteinyi residues in in the Cys-rich motif of EutT are required for activity. EutT has a putative HX₃HX₂CCX₃C motif that resembles the cytochrome oxidase CCX₃C dicopper binding site or a 4Fe/4S cluster that was recently identified in Bacillus megaterium CbiX protein (Fig. 4) (28). This cysteine-rich motif of EutT was targeted for site-directed mutagenesis to determine whether cysteinyi residues Cys79, Cys80 and Cys83 were 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 EutTC⁷⁹A, EutTC⁸⁰A or EutTC⁸³A proteins complemented a S. enterica cobA eutT strain JE7180 during growth on ethanolamine (data not shown). Variant proteins were overproduced, purified using the protocol for the EutT WT protein, and their adenosyltransferase activity was assayed in vitro (Table 2). EutTC⁷⁹A was the most active of the three variants tested, with 20% of the wild-type activity detected in the detergent-soluble fraction. In contrast, EutTC⁸⁰A and EutTC⁸³A proteins retained very little activity (0.6% and 1.2% activity, respectively). Mutant EutT proteins were predominantly found in the detergent-insoluble pellet, likely misfolded and aggregated (Table 2 ). We propose that residue Cys79 might be more important for structural integrity than residues Cys80 and Cys83.

Substrate specificity. Nucleotide substrate. Under conditions where Cbl was saturating, the Kₘ and kₐ₅ for ATP were measured at 10 µM and 0.03 s⁻¹, respectively (Fig. 5A); Vₕ₅ 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 (sp. act. = 42 vs. 36 nmol AdoCbl min⁻¹ mg⁻¹ of protein). Recognition of NTPs by EutT was unlike that of CobA or PduO (2,10). Not surprisingly, ATP was the preferred nucleotide substrate (Fig. 6A), but there were several differences between CobA and EutT in terms of the nucleotide substrate each enzyme can use. EutT retained reasonable activity (31%) when dATP was the substrate, while CobA does use dATP as substrate at all. EutT retained 38% activity when CTP was used as substrate, while CobA can use CTP 1.5-fold better than ATP. EutT activity was poor (<10%) when GTP, UTP and ITP, whereas CobA can use the latter 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 does use ADP as substrate.

Corrinoid substrate. We also determined the kinetic parameters of EutT for Cbl under conditions where MgATP was saturating. Under such conditions, $K_m = 4.1 \mu M$, $K_{app} = 0.06 \text{ s}^{-1}$, and $V_{max} = 105 \text{ nM min}^{-1}$ (Fig. 5B). In vitro, EutT adenosylated cobinamide (Cbi), an incomplete corrinoid lacking the DMB lower ligand. When Cbi was used as substrate, the specific activity of the enzyme (13 nmol AdoCbi min⁻¹ mg⁻¹ of protein) was 21% of the one measured when Cbl was the substrate (sp. act. = 62 nmol AdoCbl min⁻¹ mg⁻¹ of protein), suggesting a role for the nucleotide loop in substrate binding. Despite its in vitro activity, a cobA mutant strain (JE7172, Table 1) expressing the eutT* allele from a plasmid failed to grow on ethanolamine or glycerol as sole carbon sources when Cbi was provided in the medium. Clearly, the rate of Cbi 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 the Cbl-dependent methionine synthase (MetH) enzyme.

EutT cleaves PPPi into PPi and Pi. To date, corrinoid adenosyltransferases (10,29) and SAM synthase (30) are the only enzymes known to release triplyphosphate (PPPi) as a reaction by-product without cleaving it to pyrophosphate (PP) and orthophosphate (P). CobA is strongly inhibited by PPP, (10), and PduO is not (2). EutT was slightly inhibited by PPP, (15%) or PP, (20%) when each compound was present at 10-fold the concentration of ATP in the reaction mixture. P, did not inhibit EutT activity (Fig. 6B). Since PPP, only slightly inhibited EutT, we investigated whether EutT cleaved PPPi. For this purpose, we used 31P-NMR spectroscopy to determine if PPP, PP, or Pi were stable reaction by-products. NMR data showed that while ATP and PPP, were present in the complete reaction (Fig. 7B), PP, and Pi peaks were enhanced. When Cbl was omitted from the reaction mixture, only PPP, signals were observed (Fig. 7A). Therefore, EutT (unlike CobA and PudO) cleaved PPPi to PPi and Pi, and cleaved ATP to Ado and PPPi 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 cobA mutant background, each of these deletions was tested for their ability to affect EutT-dependent growth on ethanolamine. If eutP, eutQ, or their combination were required for cob(II)bilamin \( \rightarrow \) cob(I)bilamin reduction, mutant strains lacking these functions would be unable to grow on ethanolamine as carbon and energy source, i.e. the phenotype would be identical to that of the cobA eutT strain (JE7180). If EutP and/or EutQ proteins were not required for other metabolic functions, lack of these functions would not affect growth on ethanolamine when wild-type cobA or eutT genes were present on the chromosome. A eutQ cobA strain (JE8819) displayed a slightly reduced growth rate on ethanolamine relative to the eutQ cobA strain (JE1293) (generation time = 0.26 vs 0.23 doublings h⁻¹). However, this minor effect of the eutQ mutation on ethanolamine metabolism was independent of cobalamin adenosylation because a eutQ cobA strain (JE8816) also had a slightly reduced growth rate compared to wild-type strain (TR6583) (generation time = 0.24 vs 0.29 doublings h⁻¹). However, this minor effect of the eutQ mutation on ethanolamine metabolism was independent of cobalamin adenosylation because a eutQ cobA strain (JE8816) also had a slightly reduced growth rate compared to wild-type strain (TR6583) (generation time = 0.24 vs 0.29 doublings h⁻¹). A eutP cobA strain (JE8818) grew as well as 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 were required for EutT function.

Based on the above data, we concluded that neither EutP nor EutQ were required for EutT function.
Candidates outside the eut operon. Given the highly hydrophobic nature of the EutT protein, we postulated that a plausible source of electrons for EutT function could be the electron transport system (ETS). Various electron donors were tested for the ability to allow AdoCbl production as a function of the presence of unresolved lysate, cob(III)balamin, ATP and Mn²⁺. Lysate from cells expressing eutT from an overexpression vector supported the conversion of cob(III)balamin to AdoCbl when FMN or 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 did not require interactions with other Eut proteins.

AdoCbl was not synthesized from cob(III)balamin when purified CHAPS/EutT protein 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 interacted with EutT.

The electron transfer proteins flavodoxin (FldA) and the 2Fe/2S ferredoxin (Fdx) were tested for their ability to reduce cob(II)balamin in the active site of purified CHAPS/EutT enzyme. The S. enterica Ferredoxin(flavodoxin):NADP(H) reductase (Fpr) enzyme was used to reduce FldA and Fdx. While activity was detectable, it was only <1% of the activity measured when Ti(III)citrate was used to reduce Eut (sp. act. = 0.49 vs. 61 nmol AdoCbl min⁻¹ mg⁻¹ of protein, respectively). At present, we cannot rule out the participation of FldA or Fdx in EutT function.

**DISCUSSION**

The three classes of CA enzymes 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 than 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 PPi and adenosine when Cbl is absent from the active site. Unlike CobA or PduO, EutT cleaves PPi to P₁ and P₂, explaining why PPi is a poor inhibitor of its adenosyltransferase activity. The elucidation of three-dimensional EutT structures could provide further mechanistic insights on 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 eut 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 like S. enterica and E. coli, both of which maintain eutT within the eut operon.

**Possible means for the generation of the cob(II)balamin 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)balamin to cob(I)balamin may reflect limited productive interactions between Fdx or FldA and EutT due to the detergent needed to solubilize EutT. The fact that some activity was detected with FldA and Fdx warrants further investigation. On the other hand, 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)balamin →
The function of the EutP,Q 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 (FMNH₂, FADH₂) 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 CFE. 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.
FOOTNOTES.
§ Present address, Department of Microbiology, University of Illinois-Urbana, Urbana, IL 61801.
* This work was supported by grant R01 GM40313 from the NIH to J.C.E.-S. N.R.B. is a Howard Hughes Predoctoral Fellow. The authors acknowledge the NMRFAM at the University of Wisconsin-Madison for obtaining 31P-NMR spectra.

Abbreviations used are: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; TrisCl, tris(hydroxymethyl)aminomethane chloride; ATP, adenosine triphosphate; ADP, adenosine diphosphate, AMP, adenosine monophosphate; GTP, guanosine triphosphate; CTP, cytosine triphosphate; UTP; uracyl triphosphate; PPP, triphosphate; PPi, pyrophosphate; Pi, orthophosphate; KPi, potassium phosphate; Cbl, cobalamin; AdoCbl, adenosylcobalamin; HOCbl, hydroxocobalamin; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonylfluoride; IPTG, isopropylthio-b-D-galactopyranoside; H6Fdx, ferredoxin with a hexahistidine tag fused at its N terminus; FldAH6, flavodoxin A with a hexahistidine tag fused at its C terminus; FPLC, fast protein liquid chromatography.

REFERENCES
Table 1. Strains and plasmids used in this study. Unless otherwise indicated, all strains and plasmids were constructed during the course of this work.

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli</td>
<td>BL21 (λDE3) F' dcm ompT hsdS(rB mB) gal λ(DE3)</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>S. enterica</td>
<td>TR6583</td>
<td>metE205 ara-9</td>
</tr>
<tr>
<td>Derivatives of TR6583</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JE1096</td>
<td>cobA343::MudI1734(kan^)a</td>
<td>Lab collection</td>
</tr>
<tr>
<td>JE1293</td>
<td>cobA366::Tn10(d-cat^)</td>
<td>Lab collection</td>
</tr>
<tr>
<td>JE8292</td>
<td>cobA343::MudI1734(kan^) zfa3648<em>Tn10</em>zfa3649 (Δeut)</td>
<td>Lab collection</td>
</tr>
<tr>
<td>JE7172</td>
<td>cobA366::Tn10(d-cat^) / pEUT7</td>
<td>Lab collection</td>
</tr>
<tr>
<td>JE7179</td>
<td>cobA366::Tn10(d-cat^) eutE18::MudI1734(ΔeutT)</td>
<td>Lab collection</td>
</tr>
<tr>
<td>JE7180</td>
<td>cobA366::Tn10(d-cat^) eut1141(ΔeutQ)</td>
<td>Lab collection</td>
</tr>
<tr>
<td>JE8815</td>
<td>eutP1168 (ΔeutP)^b</td>
<td>Lab collection</td>
</tr>
<tr>
<td>JE8816</td>
<td>eutQ1169 (ΔeutQ)^b</td>
<td>Lab collection</td>
</tr>
<tr>
<td>JE8817</td>
<td>eutPQ1170 (ΔeutPQ)^b</td>
<td>Lab collection</td>
</tr>
<tr>
<td>JE8818</td>
<td>cobA343::MudI1734(ΔeutP) eutP1168 (ΔeutT)</td>
<td>Lab collection</td>
</tr>
<tr>
<td>JE8819</td>
<td>cobA343::MudI1734(ΔeutP) eutQ1168 (ΔeutQ)</td>
<td>Lab collection</td>
</tr>
<tr>
<td>JE8820</td>
<td>cobA343::MudI1734(ΔeutP) eutPQ1169 (ΔeutPQ)</td>
<td>Lab collection</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEUT7</td>
<td>bla^ P_arABAD=eutT^</td>
<td>EutT^WT</td>
<td>(3)</td>
</tr>
<tr>
<td>pEUT36</td>
<td>bla^ eutF1171</td>
<td>EutT^C79A</td>
<td></td>
</tr>
<tr>
<td>pEUT37</td>
<td>bla^ eutF1172</td>
<td>EutT^C80A</td>
<td></td>
</tr>
<tr>
<td>pEUT38</td>
<td>bla^ eutF1173</td>
<td>EutT^C83A</td>
<td></td>
</tr>
<tr>
<td>pEUT55</td>
<td>bla^ P_arABAD=eutP^</td>
<td>EutP^WT</td>
<td></td>
</tr>
<tr>
<td>pEUT56</td>
<td>bla^ P_arABAD=eutQ^</td>
<td>EutQ^WT</td>
<td></td>
</tr>
<tr>
<td>pEUT57</td>
<td>bla^ P_arABAD=eutPQ^</td>
<td>EutPQ^WT</td>
<td></td>
</tr>
<tr>
<td>pEUT58</td>
<td>bla^ P_arABAD=eutPQT^</td>
<td>EutPQT^WT</td>
<td></td>
</tr>
<tr>
<td>pFPR1</td>
<td>bla^ fpr^</td>
<td>Fpr^WT</td>
<td>Lab collection</td>
</tr>
<tr>
<td>pFLDA4</td>
<td>bla^ fldA^</td>
<td>FlmA^H6</td>
<td>(3)</td>
</tr>
<tr>
<td>pFDX1</td>
<td>bla^ fdx^</td>
<td>H1Fdx</td>
<td></td>
</tr>
</tbody>
</table>

^a Referred to in the text as MudJ (32).

^b In-frame deletion constructed using described methodology (33).
Table 1s. List of plasmids and primers used to construct them.

<table>
<thead>
<tr>
<th>Plasmid (Genotype, protein)</th>
<th>Primers</th>
</tr>
</thead>
</table>
| pEUT12 (P\textsubscript{T\textgamma}-eutT\textsuperscript{+}) (EutT\textsuperscript{WT}) | EutTNdeIf (5’GGCCTGCAAACTTGCAATCCATATGAACG3’)  
EutTXhoIr2 (5’GGGCCGGCTCAACTGAGTGGCCTCTCCAACCGTTG3’) |
| pEUT25 (P\textsubscript{T\textgamma}-eutT1165) (EutT\textsuperscript{Clf}) | EutTC79Af (5’GGGCCTGCAAACTTGCAATCCATATGAACG3’)  
EutTC79Ar (5’GGGCCGGCTCAACTGAGTGGCCTCTCCAACCGTTG3’) |
| pEUT26 (P\textsubscript{T\textgamma}-eutT1166) (EutT\textsuperscript{Clh}) | EutTC80Af (5’GGGCCGGCTCAACTTGCAATCCATATGAACG3’)  
EutTC80Ar (5’GGGCCGGCTCAACTGAGTGGCCTCTCCAACCGTTG3’) |
| pEUT27 (P\textsubscript{T\textgamma}-eutT1167) (EutT\textsuperscript{Cln}) | EutTC83Af (5’GGGCCGGCTCAACTTGCAATCCATATGAACG3’)  
EutTC83Ar (5’GGGCCGGCTCAACTGAGTGGCCTCTCCAACCGTTG3’) |
| pEUT35 (\textsuperscript{ParaBAD}- eutT1165) (EutT\textsuperscript{Clf}) | EutTEcoRIf (5’GTACGTCGCTGGAATTCAAACTGGC3’)  
EutTXbaIr2 (5’GGGCATCTAGAAAGACGACTCTTGCC3’) |
| pEUT36 (\textsuperscript{ParaBAD}- eutT1165) (EutT\textsuperscript{Cln}) | Same as pEUT35 |
| pEUT37 (\textsuperscript{ParaBAD}- eutT1165) (EutT\textsuperscript{Cln}) | Same as pEUT35 |
| pEUT55 (\textsuperscript{ParaBAD}-eutP\textsuperscript{+}) | EutP\textsubscript{Xba}Ir (5’GGGCGCGTTCAACTGAGTGGCCTCTCCAACCGTTG3’)  
EutP\textsubscript{Xba}Ir2 (5’GGGCCGGCTCAACTGAGTGGCCTCTCCAACCGTTG3’) |
| pEUT56 (\textsuperscript{ParaBAD}-eutQ\textsuperscript{+}) | EutQEcoRIf (5’AAAAAAGTCGACTCATACGGATTGCCAGTTTG3’)  
EutQ\textsubscript{Sal}Ir (5’AAAAAAGTCGACTCATACGGATTGCCAGTTTG3’) |
| pEUT57 (\textsuperscript{ParaBAD}-eutPQ\textsuperscript{+}) | EutP\textsubscript{Xba}Ir2 (5’GGGCCGGCTCAACTGAGTGGCCTCTCCAACCGTTG3’)  
EutP\textsubscript{Xba}Ir2 (5’GGGCCGGCTCAACTGAGTGGCCTCTCCAACCGTTG3’) |
| pEUT58 (\textsuperscript{ParaBAD}-eutPQT\textsuperscript{+}) | EutP\textsubscript{Xba}Ir2 (5’GGGCCGGCTCAACTGAGTGGCCTCTCCAACCGTTG3’)  
EutP\textsubscript{Xba}Ir2 (5’GGGCCGGCTCAACTGAGTGGCCTCTCCAACCGTTG3’) |
| pFDX1 (pET-16b fdx\textsuperscript{+}) (H\textsubscript{6}Fdx) | Sefdx\textsubscript{Nde}If (5’TTTTTTCTATATGCGCCGATTTTCTTGCC3’)  
Sefdx\textsubscript{Bam}Hl (5’TTTTTTCTATATGCGCCGATTTTCTTGCC3’) |
Table 2. ATP:Cob(I)alamin adenosyltransferase assays with mutant EutT proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fraction</th>
<th>Specific Activity$^a$</th>
<th>%WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>CHAPS soluble</td>
<td>61 ± 0.36</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Whole lysate</td>
<td>1.4 ± 0.25</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CHAPS soluble</td>
<td>12 ± 1.5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Insoluble fraction$^b$</td>
<td>14 ± 0.49</td>
<td>23</td>
</tr>
<tr>
<td>C79A</td>
<td>Whole lysate</td>
<td>0.04 ± 0.06</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td></td>
<td>CHAPS soluble</td>
<td>0.37 ± 0.11</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Insoluble fraction$^b$</td>
<td>0.71 ± 0.61</td>
<td>1</td>
</tr>
<tr>
<td>C80A</td>
<td>Whole lysate</td>
<td>0.37 ± 0.02</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>CHAPS soluble</td>
<td>0.75 ± 0.02</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Insoluble fraction$^b$</td>
<td>0.28 ± 0.04</td>
<td>0.5</td>
</tr>
</tbody>
</table>

$^a$ The average of three determinations using Ti(III)citrate as reductant.

$^b$ Measured after solubilization with CHAPS.
FIGURE LEGENDS

Figure 1. Contributions of the CobA and EutT ATP-Co(I)rrinoid adenosyltransferases to the pool of AdoCbl in S. enterica.

Figure 2. Expression and isolation of EutT<sup>WT</sup> protein. Proteins from cell fractions were separated by SDS-PAGE (25). Numbers on the left of the gel represent protein mass markers (kDa).

Figure 3. Metal requirements and Ni<sup>2+</sup> inhibition of EutT adenosyltransferase activity. Panel A shows the enhancement of cobalamin adenosyltransferase activity as a function of added Mn<sup>2+</sup> ions. Panel B shows EutT activity inhibition by Ni<sup>2+</sup> 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 (bold) and two histidines (outlined) that seemed reminiscent of the CCX<sub>2</sub>C dicopper binding motif of cytochrome oxidase and/or the MXCX<sub>2</sub>C 4Fe/4S motif of B. subtilis CbiX. Residues C79, C80 and C83 of EutT were changed to alanine to assess their importance in EutT function.

Figure 5. Kinetics of ATP and Cbl binding to EutT. Panel A. Cbl was held at 50 µM while ATP concentration was varied. The <i>K</i><sub>m</i><sub>ATP</sub> = 10 µM, <i>k</i><sub>cat</sub><sub>ATP</sub> = 0.03 s<sup>-1</sup> and the <i>V</i><sub>max</sub> = 54 nmoles min<sup>-1</sup> mg<sup>-1</sup>. In B, ATP was held at 500 µM while Cbl concentration was varied. The <i>K</i><sub>m</i><sub>Cbl</sub> = 4.1 µM, <i>k</i><sub>cat</sub><sub>Cbl</sub> = 0.06 s<sup>-1</sup>, and <i>V</i><sub>max</sub> = 105 nmoles min<sup>-1</sup>. Insets show Lineweaver-Burk plots.

Figure 6. NTP substrate analogs and phosphate product inhibition of EutT. Panel A. NTPs tested in lieu of ATP; all NTPs were at 0.5 mM. Panel B. PPP<sub>α</sub>, PP, or P, were added to 10X the amount of ATP present in the reaction to assess product inhibition. For ATP, the sp. act = 61 nmol min<sup>-1</sup> mg<sup>-1</sup>.

Figure 7. <sup>31</sup>P-NMR spectra of EutT reaction products. Panel A. Reaction mixture lacking Cbl. Panel B. Complete reaction mixture containing EutT, ATP and Cbl. Panels C, D. PPP, and ATP standards, respectively. Panel E. Mixture of ATP, PPP<sub>α</sub>, P<sub>γ</sub>, P, 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 µg of total protein was used in adenosylation assays. Product was detected by change in the absorbance at 525 nm after photolysis. pVOC, vector-only control; pEUT7, <i>p</i>eutT<sup>+</sup>. Several culture media were tested. Cell-free extracts of strains used in these studies were (from left to right): strain JE1293 (cobA eutT<sup>+</sup>) grown in NCE minimal medium containing ethanolamine (30 mM), and HOCbl (1mM); strains JE7204 (cobA eutT<sup>+</sup> / pVOC) and JE7205 (cobA eutT<sup>+</sup> / p<sub>eutT</sub>) grown in NCE minimal medium containing ethanolamine (30 mM) AdoCbl (1 mM) and L(+)-arabinose (200 mM); strains JE8941 (cobA eut / pVOC) and JE8942 (cobA eut / p<sub>eutT</sub>) grown in NCE minimal 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 for faster growth and higher yield. In all cases, reaction mixtures contained FMN (50 µM), NADH (1 mM) and ATP (1 mM).
Figure 1, Buan & Escalante-Semerena

ATP:Co(I)rrinoid adenosyltransferase (CobA)

Co(I)rrinoid + ATP → AdoCo(III)rrin + PPi → AdoCbl

H₂O

EutT

Pi + PPi

ATP

Co(I)Cbl

Co(III)Cbl

Co(I)Cbl

Co(II)Cbl

e⁻
Figure 2, Buan & Escalante-Semerena
Specific Activity
AdoCbl nmoles min^{-1} mg^{-1}

[Mn^{2+}] mM

Specific Activity
AdoCbl nmoles min^{-1} mg^{-1}

[Ni^{2+}] mM

Figure 3, Buan & Escalante-Semerena
Figure 4, Buan & Escalante-Semerena

EutT PQP VHGLT SSDTH PQACC CEL CRQPV VKKP
CbiX MNC DTC
Figure 5, Buan & Escalante-Semerena

A

Specific Activity
AdoCbl nmoles min⁻¹ mg⁻¹

[ATP] mM

B

Specific Activity
AdoCbl nmoles min⁻¹ mg⁻¹

[Cbl] mM
Figure 6, Buan & Escalante-Semerena

A

Specific Activity
Ncbl nmoles min⁻¹ mg⁻¹

Substrate (0.5 mM)

B

% Specific Activity
AdoCbl nmoles min⁻¹ mg⁻¹

Phosphate (1 mM)

ATP, ADP, AMP, dATP, GTP, CTP, UTP, ITP, dGTP, dCTP, dUTP, dAdo
Figure 7, Buan & Escalante-Semerena

A
Incomplete reaction:
EutT
ATP

B
Full reaction:
EutT
ATP
HOCbl

C
PPPi only

D
ATP only

E
Mix:
ATP
PPPi
PPI
Pi
HOCbl

ppm 5 0 -5 -10 -15 -20 -25
Figure 8, Buan & Escalante-Semerena

AdoCbl (nmol)
Purification and initial biochemical characterization of the ATP:co(I)alamin adenosyltransferase (EutT) enzyme of salmonella enterica
Nicole R. Buan and Jorge C. Escalante-Semerena

J. Biol. Chem. published online April 24, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M603069200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2006/06/15/M603069200.DC1