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Spectroscopic Studies of the EutT Adenosyltransferase from *Salmonella enterica*: Evidence for a Tetrahedrally Coordinated Divalent Transition Metal Cofactor with Cysteine Ligation

Ivan G. Pallares^a, Theodore C. Moore^b, Jorge C. Escalante-Semerena^b, and Thomas C. Brunold^{a,*}

^aDepartment of Chemistry University of Wisconsin-Madison, Madison WI 53706

^bDepartment of Microbiology, University of Georgia, Athens, GA 30602

Abstract

The EutT enzyme from *Salmonella enterica*, a member of the family of ATP:cobalt(I) corrinoid adenosyltransferase (ACAT) enzymes, requires a divalent transition metal ion for catalysis, with Fe(II) yielding the highest activity. EutT contains a unique cysteine-rich HX₁₁CCX₂C(83) motif (where H and the last C occupy the 67th and 83rd positions, respectively, in the amino acid sequence) not found in other ACATs and employs an unprecedented mechanism for the formation of adenosylcobalamin (AdoCbl). Recent kinetic and spectroscopic studies of this enzyme revealed that residues in the HX₁₁CCX₂C(83) motif are required for the tight binding of the divalent metal ion and are critical for the formation of a four-coordinate (4c) cob(II)alamin [Co(II)Cbl] intermediate in the catalytic cycle. However, it remained unknown which, if any, of the residues in the HX₁₁CCX₂C(83) motif bind the divalent metal ion. To address this issue, we have characterized Co(II)-substituted wild-type EutT (EutT^{WT}/Co) by using electronic absorption, electron paramagnetic resonance, and magnetic circular dichroism (MCD) spectroscopies. Our results indicate that the reduced catalytic activity of EutT^{WT}/Co relative to the Fe(II)-containing enzyme arises from the incomplete incorporation of Co(II) ions and, thus, a decrease in the relative population of 4c Co(II)Cbl. Our MCD data of EutT^{WT}/Co also reveal that the Co(II) ions reside in a distorted tetrahedral coordination environment with direct cysteine sulfur ligation. Additional spectroscopic studies of EutT/Co variants possessing a single alanine substitution at either the His67, His75, Cys79, Cys80, or Cys83 position indicate that Cys80 coordinates to the Co(II) ion, while the additional residues are important for maintaining the structural integrity and/or high affinity of the metal binding site.

Graphical abstract

*Corresponding author. 1101 University Ave, Madison, WI 53706, Phone: (608) 265-9056, Fax: (608) 262-6143, brunold@chem.wisc.edu.

SUPPORTING INFORMATION

Additional information about the samples used in this study, tables summarizing the results obtained from fits of the VTVH-MCD data, and additional Abs, MCD, and EPR spectra of Co(II)Cbl in the presence of EutT^{WT}/Co and of EutT^{WT}/Co under different conditions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

ATP:coalt(I) corrinoid adenosyltransferase (ACAT) enzymes serve a critical role in the biosynthesis of adenosylcobalamin (AdoCbl, also known as coenzyme B₁₂).^{1,2} While notably absent in some higher eukaryotes such as plants and fungi, these enzymes are found in several domains of life where they catalyze the formation of the unique Co–C bond of AdoCbl by transferring the adenosyl (Ado) moiety of an ATP molecule to a cobalt(I) corrinoid (hereafter referred to as Co(I)rrinoid) “supernucleophile” transiently generated in their active sites.³ ACATs are thus responsible for maintaining the supply of AdoCbl required during metabolism, using corrinoid precursors salvaged from external sources (such as vitamin B₁₂) or, in the case of certain bacteria, synthesized *de novo*. To date, three evolutionary unrelated families of ACATs have been identified and classified according to their *in vivo* roles in *Salmonella enterica* sv Typhimurium LT2 as CobA, PduO, and EutT.^{4–6} For CobA^{7–9} and PduO, the latter of which is homologous to the human ACAT,^{10–13} extensive spectroscopic, kinetic, and structural studies have provided insights into how these enzymes accomplish the thermodynamically challenging reduction of the cobalt(II) corrinoid (hereafter referred to as Co(II)rrinoid) substrate that precedes the adenosylation step. Initial spectroscopic evidence indicated ACATs generate an essentially square-planar, four-coordinate (4c) species via removal of the axial ligand from the Co(II)rrinoid substrate, which has been estimated to increase the reduction midpoint potential of the Co(II)/Co(I)rrinoid couple by ~250 mV.^{10,14} X-ray crystal structures of CobA from *Salmonella enterica* (*SeCobA*) and PduO from *Lactobacillus reuteri* (*LrPduO*) in complex with Co(II)Cbl and MgATP confirmed the formation of a 4c Co(II)Cbl intermediate and revealed that a phenylalanine residue, which is part of a “wall” of hydrophobic residues, occupies the position where the 5,6-dimethylbenzimidazole (DMB) ligand of Co(II)Cbl is present in the free cofactor. Though electron density associated with the DMB was not observed in these crystal structures, the intermolecular loop that connects this moiety to the corrin ring was found to be oriented away from the active site.^{11,15} Spectroscopic characterization of enzyme variants revealed that the driving force for the displacement of the DMB ligand is provided by the formation of favorable interactions among active site residues when 4c Co(II)Cbl is generated.^{16,17}

Although the three-dimensional structure of EutT is currently unavailable, the primary sequence of this class of enzymes contains a partially conserved HX₁₁CCX₂C(83) motif (where H and the last C occupy the 67th and 83rd positions, respectively, in the amino acid

sequence) that has no counterpart in the other known ACAT families. Spectroscopic and kinetic characterizations of EutT variants with a single alanine substitution within this motif indicated that these residues are critical for the uptake of divalent metal ions [M(II)] and the formation of 4c Co(II)Cbl.^{18,19,35} For example, EutT^{C80A} was found to be unable to convert Co(II)Cbl to AdoCbl both *in vivo* and *in vitro*, which has been shown in a subsequent spectroscopic study to reflect the inability of this variant to bind Co(II)Cbl.¹⁸ Similarly, EutT^{C83A} and EutT^{H67A} did not support ethanolamine catabolism, though low *in vitro* activity was observed. In contrast, the EutT^{C79A} variant retained most of the activity displayed by the wild-type enzyme. Importantly, the activities of these variants appear to be directly linked with the degree of metal incorporation into the HX₁₁CCX₂C(83) motif. While EutT^{C79A} and EutT^{H67A} bind Zn(II) and Fe(II), albeit at lower levels relative to EutT^{WT}, both EutT^{C80A} and EutT^{C83A} only incorporate trace amounts of Zn(II) and fail to bind Fe(II).¹⁹ Thus, the HX₁₁CCX₂C(83) motif clearly plays a significant role in modulating the activity of EutT; however, it remains unknown how residues in this motif interact with the Co(II)Cbl and ATP substrates, or how they assist in the binding of divalent transition metal ions.

The similarity of the HX₁₁CCX₂C(83) primary sequence motif of EutT to that of the 4Fe/4S cluster motif found in the CbiX cobaltochelatase from *B. subtilis* initially led to the proposal that EutT was capable of cobalamin reductase activity.⁴ However, magnetic circular dichroism (MCD) spectra obtained for EutT reconstituted with Fe(II) (EutT^{WT}/Fe) lack features in the 10,000 to 35,000 cm⁻¹ energy window where contributions from 4Fe/4S clusters and related species are usually found.^{18,20,21} Further evidence against the presence of an 4Fe/4S cluster in EutT is provided by the fact that the Zn(II)-bound enzyme is equally active as EutT^{WT}/Fe.

While Fe(II)- and Zn(II)-containing enzymes are difficult to characterize spectroscopically because 1) they lack any spectral features in the visible region of the electronic absorption spectrum; 2) they either have an integer spin [high-spin Fe(II)] or diamagnetic [Zn(II)] ground state; and 3) the charge transfer transitions are too high in energy for resonance Raman experiments, information about the ligand environment of the native metal ion can often be obtained by conducting spectroscopic studies of their Co(II)-substituted derivatives. The optical spectra of these Co(II) species are dominated by ligand field transitions whose energies and intensities vary substantially as a function of coordination number and geometry.²²⁻²⁶ For this reason and the fact that Co(II)-binding to the HX₁₁CCX₂C(83) motif of metal-free EutT has been shown to promote the binding of substrate Co(II)Cbl as well as to restore catalytic activity, we have characterized EutT reconstituted with Co(II) (EutT^{WT}/Co) both in the absence and the presence of Co(II)Cbl by using MCD and EPR spectroscopies. To determine which specific residues in the HX₁₁CCX₂C(83) motif might serve as ligands to the Co(II) ion, we have characterized EutT/Co variants with a single alanine substitution at either the His67, His75, Cys79, Cys80, or Cys83 position. The results obtained in this study provide unparalleled insight into the nature of the divalent metal ion binding site of EutT and the unique mechanism employed by this enzyme to generate 4c Co(II)Cbl.

MATERIALS AND METHODS

Cofactors and Chemicals

Aquacobalamin ($[\text{H}_2\text{OCbl}]\text{Cl}$) and potassium formate (HCOOK) were obtained from Sigma and used without modification. Co(II)Cbl was generated anaerobically by the addition of a $\sim 30\ \mu\text{L}$ of saturated HCOOK solution to a degassed sample ($500\ \mu\text{L}$) of aqueous H_2OCbl^+ ($5\ \text{mM}$) in a sealed vial. Formation of Co(II)Cbl was determined spectrophotometrically by monitoring the absorbance at $474\ \text{nm}$, and the reaction was deemed complete when spectroscopic features from H_2OCbl^+ were no longer visible.

Protein Preparation and Purification

EutT was obtained and purified as described elsewhere.¹⁹ Briefly, the *eutT* gene of *Salmonella enterica* sv. Typhimurium LT2 was overexpressed from a pTEV6 vector in *Escherichia coli* BL21(λDE3), which generated a fusion protein with cleavable N-terminal hexahistidine (H_6) and maltose binding protein (MBP) tags. EutT variants were generated using the QuikChange II site-directed mutagenesis kit (Stratagene). All proteins were purified using an AKTA fast protein liquid chromatograph equipped with a HisTrap nickel-affinity column (GE Healthcare). The H_6 -MBP tag was cleaved using recombinant tobacco-etch protease (rTEV).²⁷ The tag was removed from the protein solution using a HisTrap nickel-affinity column and an amylose column (New England Biolabs).

Freshly prepared EutT was re-metalated immediately prior to the performance of spectroscopic analyses. The protein was concentrated to a least $25\ \text{mg/mL}$ (Amicon Ultra, $10,000\ \text{MW}$ cut off), placed into dialysis units (D-tube mini, Novagen), and moved into an anoxic chamber. Metal-free EutT was then prepared by repeatedly dialyzed (three times for $30\ \text{min}$ ea.) against degassed buffer [HEPES ($50\ \text{mM}$, $\text{pH}\ 7$)] containing NaCl ($300\ \text{mM}$), *tris*-(2-carboxyethyl)phosphine (TCEP; $0.25\ \text{mM}$), EDTA ($2\ \text{mM}$), and low ($10\ \mu\text{M}$) or high ($1\ \text{mM}$) concentrations of MgATP . Next, apoEutT was re-metalated with Co(II) via dialysis (three times for $30\ \text{min}$ ea.) against chelex-treated buffer containing CoCl_2 ($1\ \text{mM}$) and either $10\ \mu\text{M}$ or $1\ \text{mM}$ MgATP . The EutT/ Co samples were mixed with 100% glycerol to a final concentration of $>300\ \mu\text{M}$ in 60% (v/v) glycerol. Samples were stored in airtight serum vials (Wheaton) and flash-frozen in liquid nitrogen until use. All chemicals were sourced from Sigma except where noted.

Sample Preparation—For samples containing Co(II)Cbl , purified EutT/ Co (~ 300 to $\sim 600\ \mu\text{M}$) in HEPES buffer ($50\ \text{mM}$, $\text{pH}\ 7$) containing NaCl ($300\ \text{mM}$), and TCEP ($0.25\ \text{mM}$) was mixed with Co(II)Cbl under anaerobic conditions. The samples were injected into the appropriate sample holders (MCD cells or quartz tubes) in an anoxic chamber. After removal from the chamber, Abs spectra of samples in MCD cells were collected at room temperature under an N_2 atmosphere, and all samples were immediately flash frozen and stored in liquid nitrogen.

Spectroscopy—Low-temperature Abs and MCD spectra were collected on a Jasco J-715 spectropolarimeter in conjunction with an Oxford Instruments SM-4000 8T magnetocryostat. All reported MCD spectra were obtained by taking the difference between

spectra collected with the magnetic field oriented parallel and antiparallel to the light propagation axis to remove contributions from the natural CD and glass strain. If appropriate, the temperature-independent contributions to a given MCD spectrum were removed by subtracting the spectrum obtained at 25 K from the low-temperature spectrum.

Variable-temperature variable-field (VTVH)-MCD data were obtained at three different wavelengths corresponding to the peak positions of the most intense features in the MCD spectrum of EutT^{WT}/Co. The experimental data were fit as described elsewhere.²⁸ X-band EPR spectra were obtained by using a Bruker ESP 300E spectrometer in conjunction with an Oxford ESR 900 continuous-flow liquid helium cryostat and an Oxford ITC4 temperature controller. The microwave frequency was measured with a Varian EIP model 625A CW frequency counter. All spectra were collected using a modulation amplitude of 10 G and a modulation frequency of 100 kHz. EPR spectral simulations were performed using the SIMPOW6 program.²⁹

RESULTS

Wild-type EutT/Co enzyme

Previous studies of EutT have focused on the Zn(II)- and Fe(II)-bound forms of the enzyme (EutT/Zn and EutT/Fe respectively).^{4,18,19,35,55} Wild-type EutT/Fe (EutT^{WT}/Fe) was deemed to be the most physiologically relevant species, as EutT from anoxic crude extracts binds iron, and under anoxic conditions the specific activity of purified EutT is twice as large as that of EutT^{WT}/Zn.¹⁹ However, besides a small difference in their relative populations, the four-coordinate (4c) Co(II)Cbl species generated in the active sites of EutT^{WT}/Zn and EutT^{WT}/Fe in the presence of excess MgATP are spectroscopically indistinguishable, suggesting that Zn(II) substitution minimally affects the Co(II)/Co(I)Cbl reduction step.¹⁸

The MCD spectrum of Co(II)Cbl in the presence of EutT^{WT}/Co and excess MgATP is similar, but not identical to the one reported for Co(II)Cbl bound to EutT^{WT}/Fe in complex with MgATP.^{18,35} In the former spectrum (Figure 1, A), a sharp positive peak is observed at 12 200 cm⁻¹ along with additional features at 19 000 and 20 400 cm⁻¹ that are not present in the MCD spectrum of free Co(II)Cbl (Figure 1, B). These features (referred to as the δ -, λ -, and σ -bands, respectively) are characteristic of 4c Co(II)Cbl.¹⁷ However, several additional weak MCD features are observed on the high-energy side of the δ -band that have no counterparts in the MCD spectra of other 4c Co(II)corrinoid species. These features are attributed to the high spin Co(II) center that is present in EutT^{WT}/Co (*vide infra*).

While the MCD spectroscopic signatures of the 4c Co(II)Cbl fraction generated in the EutT^{WT}/Co active site indicate that this species is present in a very similar conformation as that formed in EutT^{WT}/Fe, a comparison of the relative intensities of the δ -band indicates that the 5c $\rightarrow$ 4c Co(II)Cbl conversion yield decreases by ~44% upon substitution of Co(II) for Fe(II). Accordingly, the remaining MCD features in the 14 000 – 21 000 cm⁻¹ range, which arise from the fraction of base-on Co(II)Cbl free in solution, are considerably more intense in the presence of EutT^{WT}/Co than EutT^{WT}/Fe (Figure S1). These findings indicate that a significant fraction of Co(II)Cbl does not bind to EutT^{WT}/Co, suggesting that the substitution of Co(II) for Fe(II) leads to a decreased Co(II)Cbl binding affinity of EutT^{WT}.

As the degree of metal incorporation has been shown to correlate with the relative population of Co(II)Cbl bound to EutT^{WT}/Fe,¹⁸ it is reasonable to assume that the larger fraction of unbound Co(II)Cbl in the presence of EutT^{WT}/Co stems from incomplete Co(II) incorporation.

Although the MCD spectrum of Co(II)Cbl in the presence of EutT^{WT}/Co and excess MgATP appears to lack any contributions from base-off Co(II)Cbl (a five-coordinate species in which a water molecule rather than the DMB moiety serves as the axial ligand), features associated with this species are difficult to discern because they are obscured by contributions from base-on, DMB-bound Co(II)Cbl. Therefore, we used EPR spectroscopy as a complementary probe of the axial ligand environment of the different Co(II)Cbl species that are present in samples of EutT^{WT}/Co and variable amounts of MgATP.

The EPR spectrum of Co(II)Cbl in the presence of EutT^{WT}/Co and minimal MgATP (<10 μ M, so as to maintain protein stability) is dominated by features from base-on Co(II)Cbl species (Figure S3A; note that under these conditions the high spin Co(II) center that is present in EutT^{WT}/Co contributes insignificantly to the EPR spectrum). However, a weak positive feature is observed at 2 600 G that is characteristic of base-off Co(II)Cbl bound to the active site of the enzyme. A quantitative analysis of this spectrum reveals that ~21% of the cofactor is present in the base-off conformation under these conditions. In the presence of a 3-fold molar excess of MgATP (Figure S3B), the relative intensities of the EPR features associated with base-off Co(II)Cbl are considerably higher, indicating that the relative population of this species has increased to ~50%. While contributions from 4c Co(II)Cbl are not readily discernible, weak features centered at 2 000 G are consistent with the presence of such a species (note that due to the extreme g anisotropy displayed by 4c Co(II)Cbl,¹⁸ the relative population of this species would have to exceed 25% to produce noticeable contributions to our EPR spectra). The remaining features in the EPR spectrum of Co(II)Cbl in the presence of EutT^{WT}/Co and a 3-fold molar excess of MgATP arise from “base-on” Co(II)Cbl species. Although these features are very similar to those displayed by Co(II)Cbl free in solution, the sharpening of the features at ~3 000 G can be attributed to the presence of an enzyme-bound base-on Co(II)Cbl species with restricted conformational freedom. Note that because the EPR spectra of the base-on and base-off Co(II)Cbl species in the EutT^{WT}/Co active site do not exhibit any dipolar broadening, it can be concluded that the divalent metal binding site is at least 10 Å away from the Co(II) ion of the corrinoid substrate.⁶⁰

In summary, our spectroscopic data indicate that Co(II)Cbl interacts with EutT^{WT}/Co in a similar manner as with the EutT^{WT}/Fe enzyme, though the fraction of Co(II)Cbl binding to the active site is lower. This difference most likely arises from non-stoichiometric Co(II) incorporation into EutT^{WT}, since the presence of a divalent metal cofactor appears to be critical for Co(II)Cbl binding to this enzyme.¹⁸ Although the binding affinity of EutT^{WT} for Co(II) is unknown, a comparison of the 5c→4c Co(II)Cbl conversion yields observed for EutT^{WT} in complex with either Co(II), Zn(II), or Fe(II) suggests that Co(II) occupies only ~56% of the active sites.¹⁸ This estimate agrees well with the results obtained in a previous biochemical characterization of EutT^{WT}, which revealed that the enzyme retains ~60% of its specific activity when Fe(II) is replaced with Co(II).¹⁹

Nature of the M(II) binding site of EutT^{WT}

As noted above, a subset of weak features in the MCD spectrum of Co(II)Cbl in the presence of EutT^{WT}/Co and excess MgATP (Figure 1, A) are unlikely to arise from the Co(II)rrinoid substrate. Consistent with this hypothesis, these same features dominate the MCD spectrum of EutT^{WT}/Co obtained in the absence of substrate (Figure 2, A). Evidence that Co(II) binds to the functionally relevant, divalent metal ion binding site of EutT is provided by the fact that in the MCD spectrum of as-isolated EutT^{WT}/Zn incubated with roughly equimolar amounts of CoCl₂, the intensities of these features are reduced by a factor of ~50 (Figure 2, B). This result indicates that less than 2% of Co(II) is able to bind to the divalent metal site of EutT^{WT}/Zn, consistent with the nearly complete incorporation of Zn(II) into this site. Notably, the intensities of the remaining MCD features between 17 000 and 26 000 cm⁻¹ vary insignificantly between EutT^{WT}/Co and EutT^{WT}/Zn incubated with CoCl₂, indicating that these features arise from Co(II) bound to a different site.

The two sets of features observed near 15 000 and 30 000 cm⁻¹ in the MCD spectrum of EutT^{WT}/Co (Figure 2, A) are reminiscent of those previously reported for Co(II)-substituted metalloproteins featuring a tetrahedral active site with at least one cysteine ligand.^{30,31,32} The low-energy features were assigned to the spin-allowed ⁴A₂ → ⁴T₁(P) ligand field (LF) transition in the parent T_d point group, while the features at higher energies were attributed to transitions containing predominantly S → Co(II) ligand-to-metal charge-transfer (LMCT) character.^{30,31} By analogy, we assign the prominent negative feature at 13 800 cm⁻¹ with a shoulder at 14 600 cm⁻¹ along with positively signed band at 15 500 cm⁻¹ in the MCD spectrum of EutT^{WT}/Co to components of the ⁴A₂ → ⁴T₁(P) LF transition of the Co(II) ion. Notably, the splitting pattern and intensity distribution of these features are very similar to those exhibited by Co(II)-substituted alcohol dehydrogenase (ADH/Co), a Zn(II)-containing enzyme *in vivo*, as well as Co(II)-substituted stellacyanin (SC/Co), a type-1 blue copper protein.^{30,31} However, the intense negatively-signed feature at 13 800 cm⁻¹ in the MCD spectrum of EutT^{WT}/Co is significantly red-shifted from its counterparts in the ADH/Co and SC/Co spectra (where this feature is observed 16 000 and 15 000 cm⁻¹, respectively). A shift of this feature to lower energy has been correlated with an increase in the number of thiolate ligands of tetrahedral Co(II) complexes,^{30,31,32} suggesting that multiple cysteine residues are present at the divalent metal site of EutT^{WT}.

The positively signed feature at 28 400 cm⁻¹ and two negative features at 30 500 and 32 700 cm⁻¹ in the MCD spectrum of EutT^{WT}/Co are similarly intense as the lower-energy bands associated with LF transitions, but their widths are significantly larger (Figure 2, A). Based on their energies and bandwidths, these near-UV features are assigned to S → Co(II) LMCT transitions. Analogous features are observed in the MCD spectra of ADH/Co and SC/Co,^{30,31} as well as Co(II)-substituted rubredoxin proteins known to contain multiple sulfur-based ligands at their active sites.³² In the case of ADH/Co, MCD features associated with S → Co(II) LMCT transitions are observed between 26 000 and 35 000 cm⁻¹ for Co(II) bound to the catalytic site (consisting of two cysteine residues, one histidine, and a solvent-derived water molecule) or the structural site (consisting of four cysteine residues), with the intensities of the features arising from the former, less symmetric site being significantly larger. In the case of SC/Co, which coordinates the metal via a single cysteine residue, two

histidines, and the oxygen atom from a glutamine residue, the $S \rightarrow \text{Co(II)}$ LMCT transitions are centered at $28\,000\text{ cm}^{-1}$ (with the lowest-energy, positively signed MCD feature peaking at $25\,500\text{ cm}^{-1}$). This feature occurs at even lower energies in the MCD spectra of the closely related, Co(II) -substituted type-1 copper proteins azurin and plastocyanin ($24\,500$ and $22\,500\text{ cm}^{-1}$ respectively). On the basis of these data and the fact that a more anionic ligation sphere will decrease the Lewis acidity of the Co(II) ion, we attribute the relatively high energies of the $S \rightarrow \text{Co(II)}$ LMCT transitions observed for $\text{EutT}^{\text{WT}}/\text{Co}$ to the presence of at least two cysteine residues at the divalent metal binding site. Although the number and identity of the additional ligand(s) remains unknown, we note that the MCD spectrum of $\text{EutT}^{\text{WT}}/\text{Co}$ is particularly similar to those of Co(II) bound to either the catalytic or structural site of ADH, both of which have been shown by X-ray crystallography to adopt a fairly regular tetrahedral geometry. Thus, it appears that the Co(II) ion of $\text{EutT}^{\text{WT}}/\text{Co}$ also resides in a weakly distorted tetrahedral coordination environment.³³

To further assess the geometry of the $\text{EutT}^{\text{WT}}/\text{Co}$ metal binding site, variable-temperature variable-field (VTVH) MCD data were collected at the peak positions of the three most intense bands arising from the $^4\text{A}_2 \rightarrow ^4\text{T}_1(\text{P})$ LF and $S \rightarrow \text{Co(II)}$ LMCT transitions (Figure 3). These data were then analyzed within the framework of the MCD theory for $S > 1/2$ systems developed by Neese and Solomon to obtain the spin-Hamiltonian parameters for the Co(II) ion of $\text{EutT}^{\text{WT}}/\text{Co}$.²⁸ As the VTVH-MCD saturation behavior depends on a total of eight different parameters, the approach employed by Fiedler et al. was used to systematically determine the spin-Hamiltonian parameters of interest, including the axial zero-field splitting parameter, D , and the rhombicity, E/D .³⁴ To reduce the number of fit parameters, an isotropic g value of 2.20 was chosen for the Co(II) ion bound to $\text{EutT}^{\text{WT}}/\text{Co}$ based on the g values reported for other high-spin, tetra-coordinate Co(II) complexes^{22,34} because the EPR spectrum of $\text{EutT}^{\text{WT}}/\text{Co}$ is very broad due, at least in part, to the fact that Co(II) not only binds to the divalent site but to at least one additional site (Figure S4; note that the fitted values for the remaining spin-Hamiltonian parameters are expected to depend only weakly on the g values). The sign and magnitude of D were then determined by systematically varying this parameter and fitting the three transition moment products to achieve the best agreement between the experimental and calculated VTVH-MCD data while keeping the remaining spin-Hamiltonian parameter (i.e., the E/D ratio) constant. For each value of D , the goodness of the fit was evaluated by computing the χ^2 value. Finally, the E/D ratio was varied from 0.00 to 0.33 for each value of D considered and the χ^2 value was used to identify the complete set of spin-Hamiltonian parameters that best reproduced our experimental VTVH MCD data. Resulting χ^2 values for each condition are reported in the supplementary information.

Fits of the VTVH MCD data obtained at $13\,810\text{ cm}^{-1}$ (724 nm), which exhibit the least amount of nesting, were relatively insensitive to the magnitude of D , although a shallow minimum for χ^2 was observed near $D = -10\text{ cm}^{-1}$ and $E/D = 0.08$. Alternatively, the VTVH MCD data obtained at $32\,630\text{ cm}^{-1}$ (306.5 nm) show significant nesting, which could only be reproduced with an E/D ratio close to zero and large negative values for D . More accurate estimates of D and E/D were obtained by adding the χ^2 values for the three individual VTVH MCD data sets for a given pair of D and E values to calculate the global χ^2 value, which reached a distinct minimum for $D = -13.0(5)\text{ cm}^{-1}$ and $E/D = 0.08(2)$. These spin-

Hamiltonian parameters are comparable to those previously determined for tetra-coordinate Co(II) sites with minor deviations from an ideal tetrahedral geometry,^{23,25,26} consistent with our MCD spectral analysis presented above.

Effect of amino acid substitutions on Co(II)-binding by EutT

Previous studies of EutT/Fe indicated that single alanine substitutions of residues in the HX₁₁CCX₂C(83) motif can drastically suppress, or completely abolish, catalytic activity. Biochemical and spectroscopic characterizations of these variants revealed a clear correlation between the degree of Fe(II) incorporation and the relative yield of 4c Co(II)Cbl formed in the enzyme active site.^{18,19,35} The C80A and C83A substitutions were found to have a particularly large negative effect on enzyme activity, as the corresponding variants are essentially unable to bind Fe(II) and, thus, Co(II)Cbl. Consistent with this finding, kinetic assays indicated that as-isolated EutT^{C80A} is completely inactive, while EutT^{C83A} is capable of very slow turnover. Thus, all currently available data strongly suggest that both C80 and C83 serve as ligands to the divalent metal ion of EutT, with C80 playing the most important role.

Our results indicate that substitutions of residues in the HX₁₁CCX₂C(83) motif also have a large effect on the degree of Co(II) incorporation into EutT (Figure 4). Consistent with residues C80 and C83 being critical for the binding of Co(II) to the enzyme, the 4 K – 25 K difference MCD (MCD) spectra of EutT^{C80A}/Co and EutT^{C83A}/Co exhibit features that are due, solely, to Co(II) bound non-specifically or to a different site (Figure 4, E and F). Alternatively, the MCD spectrum of EutT^{C79A}/Co is nearly identical to that of EutT^{WT}/Co, except for a minor (~50 cm⁻¹) blue-shift of the prominent negative feature at 14 000 cm⁻¹ (Figure 4, B). However, the intensity of this feature is reduced by ~45% from that displayed by the wild-type enzyme, indicating that the C79A substitution causes a large decrease in the affinity of EutT for Co(II), mirroring the results obtained previously for Zn(II)- and Fe(II)-binding to this variant. Based on these results, we conclude that the side chain of C79 does not coordinate to the divalent metal ion, but does play an important role in increasing the degree of metal incorporation into EutT. Intriguingly, the MCD spectra of EutT^{H67A}/Co and EutT^{H75A}/Co appear to lack the characteristic features of Co(II) bound to the divalent metal site (Figure 4, C and D), even though these variants bind an almost equimolar amount of Fe(II) ions.¹⁹ While weak features are observed in the region where the ⁴A₂ → ⁴T₁(P) LF and S → Co(II) LMCT transitions are expected to occur, the intensities of these features indicate that at most 1% of the divalent metal sites of EutT^{H67A} and EutT^{H75A} are occupied by Co(II). Because both variants remain catalytically active when reconstituted with Fe(II) or Zn(II), our results indicate that the H67A and H75A substitutions primarily affect the ability of EutT to bind Co(II).

Interaction between EutT^{WT}/Co and AdoCbl

The low-temperature Abs, CD, and MCD spectra of AdoCbl in the presence of EutT^{WT}/Co are shown in Figure 5. The lowest-energy features in the Abs and spectra observed at ~20 000 cm⁻¹ are essentially identical to those displayed by free AdoCbl. Because the energies of these features, the so-called α/β-bands, are highly sensitive to changes in the axial ligation, our spectra are consistent with an essentially unperturbed ligand environment of

AdoCbl in the presence of EutT^{WT}/Co and minimal MgATP (<10 μ M). However, the MCD spectrum of EutT^{WT}/Co + AdoCbl collected at 4.5 K, which is dominated by temperature-dependent contributions from the paramagnetic Co(II) ions bound to the divalent metal site of EutT, reveals a small blue-shift of the intense negatively-signed feature due to the $^4A_2 \rightarrow ^4T_1(P)$ transition along with minor changes in the intensities and positions of the S $\rightarrow$ Co(II) LMCT features. Interestingly, the presence of AdoCbl appears to have a much larger effect on the spectroscopic features associated with Co(II) ions bound non-specifically or to a different site. To remove the temperature-independent contributions from AdoCbl to the EutT^{WT}/Co + AdoCbl MCD spectra, the trace obtained at 25 K was subtracted from the lower-temperature data to obtain the MCD spectra shown in Figures 6 and 7. These difference spectra indicate that the spectroscopic features arising from Co(II) ions bound to the minority site(s) are replaced by a new set of bands that have no counterparts in the

MCD spectrum of EutT^{WT}/Co in the absence of AdoCbl (Figure 7). This finding indicates that a large fraction of AdoCbl is bound to EutT, despite the lack of changes observed in our spectra of AdoCbl upon the addition of EutT^{WT}/Co. Collectively, these data suggest that once AdoCbl is formed, it remains associated with EutT, albeit in a minimally perturbed, DMB-on conformation.

It is tempting to speculate that the Co(II) minority binding site of EutT/Co corresponds to the Mg(II) binding site of MgATP. In the X-ray crystal structures of *SeCobA* and *LrPduO*, the Mg(II) ion is present in an octahedral ligand environment consisting of 3 oxygen atoms from the triphosphate moiety, the carbonyl oxygen of a nearby asparagine residue, and 2 ordered water molecules. Incorporation of AdoCbl into the EutT active site will necessarily preclude the binding of MgATP, leaving the site where the Mg(II) ion would be located following the transfer of the adenosyl moiety from MgATP to Co(I)Cbl available for Co(II) to bind.

DISCUSSION

Since its initial identification as an ACAT, the EutT enzyme from *S. enterica* has been postulated to employ a novel mechanism for generating AdoCbl.^{4,35} Intriguingly, a divalent transition metal ion was found to be critical for supporting catalytic activity of this enzyme, with Fe(II) or Zn(II) incorporation yielding the highest specific activities. However, while recent experimental studies have provided insight into the unique mechanism employed by EutT for the 5c $\rightarrow$ 4c conversion of the Co(II)Cbl substrate, the nature of the divalent metal binding site remained elusive. In this study, we have used Co(II) as a spectroscopic probe of Fe(II) and Zn(II) to obtain unprecedented insight into the structure and function of the divalent metal binding site of EutT.

Nature of the divalent metal binding site of EutT

Based on our spectroscopic data obtained for EutT^{WT}/Co, the catalytically-relevant divalent metal binding site where Fe(II) and Zn(II) are expected to bind *in vivo* comprises four ligands arranged in a nearly regular tetrahedral configuration. While fits of our VTVH-MCD data yielded *D* and *ED* values that are also consistent a five-coordinate Co(II) center,^{25,26} the energies and intensities of the LF transitions favor a four-coordinate Co(II) site.^{24,36} A

comparison of the MCD spectra of EutT^{WT}/Co and other Co(II)-substituted metalloproteins indicates that two or more cysteine residues likely coordinate to the Co(II) ion in the former, as proposed previously on the basis of site directed mutagenesis studies.¹⁹ LF transitions of tetrahedral, high-spin Fe(II) complexes occur in the near-infrared (<8,000 cm⁻¹), while LMCT transitions of Fe(II) complexes with thiolate ligation are expected to occur at energies > 33 000 cm⁻¹, which explains why we were unable to observe any MCD signals for EutT^{WT}/Fe with our instrument.^{37,38}

Our data indicate that a single alanine substitution in the HX₁₁CCX₂C(83) motif can drastically reduce the affinity of EutT for Co(II), consistent with previous observations linking this motif to Zn(II) and Fe(II) incorporation.^{18,19} The MCD spectra of the various EutT/Co variants investigated suggest that residues C80 and C83 are essential for divalent metal binding, in agreement with published kinetics and spectroscopic data for the EutT^{C80A} and EutT^{C83A} variants in complex with Fe(II). Despite the close proximity of residues C79 and C80, our data indicate that the EutT^{C79A} variant binds ~55% as much Co(II) as EutT^{WT} does under the same conditions. Moreover, the MCD spectra of the protein-bound Co(II) fractions of EutT^{C79A} and the WT enzyme are superimposable, ruling out the possibility that C79 also serves as a ligand to the Co(II) ion.

The MCD spectra of the EutT^{H67A}/Co and EutT^{H75A}/Co variants lack any features associated with Co(II) bound to the divalent metal site, indicating that H67 and H75 are critical for Co(II)-binding to EutT. In contrast, both of these variants are capable of binding Fe(II) or Zn(II) and of generating significant amounts of 4c Co(II)Cbl species (~12 and ~26 % relative to EutT^{WT}/Fe in the case of EutT^{H67A}/Fe and EutT^{H75A}/Fe, respectively), which provides clear evidence against residues H67 and H75 serving as ligands to the metal center. The fact that the H67A and H75A substitutions have a particularly large effect on the yield of Co(II)-incorporation into EutT indicates that the divalent metal site binds Co(II) more weakly than Fe(II) or Zn(II), consistent with our observation that Co(II) is unable to displace Zn(II) from EutT^{WT}/Zn.

Although our spectroscopic data are most consistent with a tetrahedral geometry of the divalent metal site and the presence of multiple cysteine sulfur ligands, it is not possible to definitively establish the exact structure of this site. However, given that EutT has been shown to incorporate one Fe(II) or Zn(II) ion per protein dimer on the basis of gel filtration experiments,¹⁹ it is reasonable to assume that both EutT monomers provide ligands to a single metal site. Moreover, because the EutT^{C80A} and EutT^{C83A} variants are unable to bind any detectable amounts for Fe(II) or Co(II), we propose that the C80 and C83 residues from each of the two EutT monomers coordinate to the divalent metal ion to form an M(S_{C80})₂(S_{C83})₂ complex. Examples of tetrahedral cysteine coordination of Zn(II) are provided by Zn-finger motifs, and in many cases, Co(II) has been used as a spectroscopic probe of these sites.^{39,40} Similar to EutT, the Zn-finger motifs have a higher affinity for Zn(II) than Co(II).^{41,42} Note that because residues H67 and H75 are critical for Co(II)-binding to EutT while substitutions of these residues minimally affect Fe(II)- or Zn(II)-binding to EutT, it could be argued that in the case of Co(II)-substituted EutT, H67, H75, C80, and C83 from the same protomer are involved in Co(II) binding. However, because Fe(II) and Zn(II) likely adopt tetrahedral Cys coordination by two EutT protomers, the

Co(II) ion in one of the two protomers of EutT/Co would be expected to be in close proximity to the Co(II) ion in the other protomer. Yet, our EPR and VTVH MCD data of EutT/Co do not provide any evidence for magnetic coupling between adjacent Co(II) centers (which would lead to dipolar broadening of the EPR spectra and poor agreement between the experimental and simulated VTVH data), indicating that the Co(II) would have to be separated by at least 10 Å. Moreover, given the fact that EutT/Fe and EutT/Co generate indistinguishable 4c Co(II)Cbl species while metal-free EutT is unable to displace the axial ligand from Co(II)Cbl, Co- and Fe-binding to the divalent metal site must cause very similar protein structural changes.

Tetrahedral coordination of Fe(II) by four cysteine residues is observed for rubredoxins, a family of electron-transfer proteins commonly found in sulfur-metabolizing anaerobic organisms that cycle between the Fe(II) and Fe(III) oxidation states.^{43,44} While we propose that a similar Fe(II) binding site is present in EutT, this site appears to play primarily a structural role, promoting the binding of Co(II)Cbl to the enzyme active site, rather than a role as an electron-transfer center. In fact, exposure of EutT^{WT}/Fe to molecular oxygen results in the deactivation of the enzyme by preventing it from binding Co(II)Cbl, presumably due to loss of Fe(III) from the divalent metal site. The stability of rubredoxins in both Fe oxidation states has been shown to stem from the ability of the protein environment to retain the native fold even in the absence of Fe ions.^{45,46} Unlike rubredoxins, EutT does not retain the proper conformation for Co(II)Cbl binding in the absence of transition metal ions, so the loss of Fe(III) from the divalent metal site upon air-exposure of EutT^{WT}/Fe is not necessarily unexpected.

Divalent metal sites in corrinoid-binding enzymes

Although the other ACAT enzymes (*i.e.*, CobA and PduO) do not possess an M(S_{Cys})₄ motif similar to the one proposed for EutT, other examples of enzymes that feature an FeS center and bind corrinoid cofactors or precursors are known. The CbiX cobaltochetalase from *Bacillus megaterium*, which participates in the biosynthesis of AdoCbl via insertion of cobalt into a sirohydrochlorin precursor, was shown to contain a 4Fe/4S cluster, although the physiological role of this motif remains unknown.⁵⁰ Alternatively, corrinoid iron-sulfur proteins (CoFeSPs), which catalyze methyl-transfer reactions, are known to employ a 4Fe/4S cluster for the reactivation of their bound cobalamin cofactor following the occasional oxidation of the Co(I)Cbl intermediate during turnover.^{51,52} Finally, recent crystal structures of the AdoCbl-dependent reductive dehalogenases PceA from *Sulfospirillum multivorans* and NprdhA from *Nitratireductor pacificus* revealed two 4Fe/4S clusters ~8 Å and ~12 Å away from the Co center of the corrinoid cofactor. These clusters have been postulated to be involved in electron transfer from external reducing agents to the active site containing Co(II)Cbl, which lies buried in the enzyme core.^{53,54} Thus, the presence of a FeS motif in EutT is not unique among corrinoid-binding enzymes; however, a single Fe(S_{Cys})₄ site serving primarily a structural role is a novel feature.

Effect of metal substitution on EutT activity

Our spectroscopic data indicate that the substitution of Co(II) for Fe(II) in EutT^{WT} results in a decrease in the relative yield of 4c Co(II)Cbl by ~55%. Because the formation of 4c

Co(II)Cbl is a prerequisite for the one-electron reduction of the Co(II) ion, the relative population of Co(I)Cbl species generated in the EutT^{WT}/Co active site immediately following the addition of a reducing agent is also expected to be diminished by ~55%, consistent with the ~60% decrease in specific activity of EutT observed upon Co(II) substitution for Fe(II) under anoxic conditions.¹⁹ The MCD features associated with the 4c fraction of Co(II)Cbl bound to EutT^{WT}/Co are identical to those observed with EutT^{WT}/Zn and EutT^{WT}/Fe, while the overall shape of the MCD spectrum is nearly identical to that of EutT^{C79A}/Fe. This variant has been shown to bind ~40% less Fe(II) and generate ~60% less 4c Co(II)Cbl than EutT^{WT}/Fe under the same conditions. As metal incorporation is known to be critical for the formation of 4c Co(II)Cbl species, the decrease in the specific activity of EutT^{WT}/Co most likely stems from incomplete Co(II) binding to the divalent metal site, rather than the formation of a perturbed, non-competent active site geometry in the presence of Co(II).

Substitution of Zn(II) for Fe(II) has been shown to have no effect on the relative yield of 4c Co(II)Cbl species generated by EutT^{WT} in the presence of an excess MgATP.¹⁸ However, the specific activity of EutT^{WT}/Zn is ~50% lower than that of EutT^{WT}/Fe, indicating that the metal substitution affects later steps in the catalytic cycle. To this end, it is interesting to note that a structural comparison of the Zn(S_{cys})₄ and native Fe(S_{cys})₄ motifs in the *Clostridium pasteurianum* rubredoxin revealed that the M(II)–S(Cys) bonds are on average ~0.1 Å longer in the Zn-bound structure [2.345 vs. 2.262 Å].⁴⁹ While these differences did not result in drastic perturbations to the enzyme fold of the *C. pasteurianum* rubredoxin, small structural changes of EutT accompanying Zn(II) substitutions for Fe(II) could affect the Co–C bond formation step by altering the relative positioning of the transiently generated Co(I)Cbl supernucleophile and the 5'-carbon of co-substrate ATP. In support of this proposal, it has been shown that amino acid substitutions in the active sites of the CobA- and PduO-type ACATs aimed at altering the relative positioning of the two substrates could cause a significant decrease in catalytic activity, despite the fact that the variants retained the ability to generate sizable amounts of 4c Co(II)Cbl.^{16,17}

Role of putative M(S_{cys})₄ motif in the catalytic mechanism of EutT

Extensive kinetic and spectroscopic investigations of EutT have provided significant insights into the catalytic mechanism employed by this enzyme. *In vivo* bioassay studies revealed that EutT cannot adenosylate cob(II)inamide [Co(II)Cbi⁺], a naturally occurring Co(II)Cbl analogue that lacks the nucleotide loop and terminal DMB moiety and instead possesses a water molecule in the “lower” axial position.⁵⁵ MCD data indicated that no 4c Co(II)Cbi⁺ species is generated in the presence of EutT and excess MgATP, precluding reduction of this alternative substrate to the Co(I) “superreduced” state in the absence of non-physiological reducing agents.³⁵ Because the axial Co–OH₂ bond of Co(II)Cbi⁺ is weaker than the Co–N(DMB) bond of Co(II)Cbl, this finding indicated that EutT features a specific binding site for the nucleotide loop of Co(II)Cbl to enhance its affinity for the native substrate and promote axial ligand dissociation. In contrast, CobA- and PduO-type ACATs can adenosylate both Co(II)Cbl and Co(II)Cbi⁺ and they lack a specific DMB binding site.^{11,15,23}

A recent spectroscopic characterization of Co(II)Cbl in the presence of EutT and sub-stoichiometric amounts of MgATP disclosed the formation of sizeable amounts of an enzyme-bound base-off Co(II)Cbl species, in which the DMB moiety was replaced by a solvent-derived water molecule.¹⁸ This finding suggested that EutT binds Co(II)Cbl prior to MgATP and that in this process, EutT promotes the dissociation of the DMB moiety to generate a base-off Co(II)Cbl intermediate. In the absence of divalent metal ions (or upon oxygen exposure in the case of EutT^{WT}/Fe), no base-off Co(II)Cbl was observed, and the base-on Co(II)Cbl species did not appear to bind to the EutT active site. Because our EPR spectra of the enzyme-bound populations of Co(II)Cbl in the presence of EutT^{WT}/Co and excess MgATP (Figure S1) are essentially identical to those obtained previously for Co(II)Cbl bound to EutT^{WT}/Zn,¹⁸ it can be concluded that the Co(II) ions in the divalent metal site and the Co(II)Cbl substrate are separated by at least 10 Å.⁶⁰ On the basis of these observations, we propose that the M(S_{cys})₄ site of EutT^{WT} serves to maintain the proper architecture of the binding pocket for the nucleotide loop and DMB ligand upon Co(II)Cbl binding to the enzyme active site (Figure 8).

While the exact reason why EutT features a putative M(S_{cys})₄ site remains unclear, it is interesting to note that FeS clusters have been found to regulate gene expression under oxidative stress by serving as redox sensors of transcription enzymes.^{58,59} Given that EutT^{WT}/Fe is deactivated in the presence of molecular oxygen via oxidation of the Fe(II) ions and release of the Fe(III) ions that are formed in this process, a possible role of the M(S_{cys})₄ motif as a redox sensor seems plausible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Abs spectra collected at 4.5 K (gray traces) and variable-temperature MCD spectra at 7 T of (A) Co(II)Cbl in the presence of EutT^{WT}/Co and ~1 mM MgATP and (B) free Co(II)Cbl. MCD features arising from 4c Co(II)Cbl are labeled with Greek letters. The feature highlighted by an asterisk in the MCD spectrum in (A) at ~14 000 cm^{-1} is due to the presence of Co(II) bound to EutT.

Figure 2.

Abs spectra collected at 4.5 K (black traces) and variable-temperature MCD spectra at 7 T of (A) EutT^{WT} reconstituted with Co(II) and (B) as-purified EutT^{WT}/Zn incubated with equimolar excess of CoCl₂. Gray arrows in (A) indicate the bands at which the VTVH-MCD data shown in Figure 3 were collected.

Figure 3. VTVH-MCD data collected at 724, 679.5, and 306.5 nm for EutT^{WT}/Co (colored dots). Data were obtained at temperatures of 2.2, 4.5, 8, 15, and 25 K as a function of magnetic fields ranging from 0 to 7 T. Theoretical fits are shown as solid black lines.

Figure 4.

MCD spectra of EutT^{WT}/Co and selected variants obtained by taking the difference between spectra collected at 4.5 and 25 K. The main features associated with the primary divalent metal binding site are highlighted by a dashed vertical line (*a*). The derivative feature arising from Co(II) bound non-specifically or to a different site is also indicated (*b*).

Figure 5.

Abs (Top), CD (middle), and MCD (bottom) spectra of AdoCbl in the presence of EutT^{WT}/Co (blue) and free in solution (red). Spectra were collected at 4.5 K unless noted otherwise. The MCD spectrum of AdoCbl in the presence of EutT^{WT}/Co collected at 4.5 K (bottom, dashed blue line) was scaled by half to facilitate a comparison with the corresponding 25 K trace (bottom, solid blue line)

Figure 6.

Low-energy region of MCD spectra of (A) EutT^{WT}/Co incubated with an approximately equimolar amount of AdoCbl and (B) substrate-free EutT^{WT}/Co collected at 4.5, 8 and 15 K after subtracting the corresponding MCD spectra obtained at 25 K (MCD signal intensity decreases with increasing temperature). The positions of the main features from the primary divalent metal binding site are highlighted by dashed vertical lines.

Figure 7.

High-energy region of MCD spectra of (A) EutT^{WT}/Co incubated with AdoCbl in an approximate equimolar ratio and (B) substrate-free EutT^{WT}/Co collected at 4.5, 8 and 15 K after subtracting the corresponding MCD spectra obtained at 25 K (MCD signal intensity decreases with increasing temperature). The center position of the main derivative-shaped feature associated with Co(II) bound non-specifically or to a different site is highlighted by a dashed vertical line.

Figure 8.

Proposed mechanism for the binding of the divalent metal ion and the Co(II)Cbl and ATP substrates to EutT, based on the results obtained in the present study. (i) In the absence of the divalent transition metal cofactor, EutT is unable to bind Co(II)Cbl due, possibly, to an improper architecture of the DMB binding pocket. (ii) Binding of the divalent transition metal cofactor by the C80 and C83 residues of both protein monomers causes a reorganization of the DMB binding pocket that is lined by residues of the $HX_{11}CCX_2C(83)$ motif. (iii) The change in the architecture of the DMB binding pocket now allows Co(II)Cbl (and ATP) to bind to the enzyme active site.

EPR parameters for Co(II)Cbl in the presence of EurT^{WT}/Co. Percent contributions from individual Co(II)Cbl species to each spectrum are indicated in bold. Parameters for free Co(II)rinoids from reference 18 are shown for comparison.

Table 1

Sample	Species	% Cont.	g-values			A(⁵⁹ Co) / MHz			A(¹⁴ N) / MHz		
			g _z	g _y	g _x	A _z	A _y	A _x	A _z	A _y	A _x
A. Free Co(II)Cbl	base-on	100	2.004	2.232	2.269	303	30	40	49	49	49
	base-off	100	1.994	2.314	2.423	402	210	226	n/a	n/a	n/a
C. EurT ^{WT} /Co + Co(II)Cbl	base-on	81	2.004	2.232	2.269	303	30	40	49	49	49
	base-off	19	1.991	2.315	2.459	387	222	227	n/a	n/a	n/a
D. EurT ^{WT} /Co + Co(II)Cbl / xcs ATP	base-on	41	2.004	2.232	2.269	303	30	40	49	49	49
	base-off	38	1.991	2.315	2.459	387	222	227	n/a	n/a	n/a
	4c	21	1.800	2.573	3.610	760	615	1362	n/a	n/a	n/a