Enzyme Catalysis

Deutsche Ausgabe: DOI: 10.1002/ange.201501930
Internationale Ausgabe: DOI: 10.1002/anie.201501930

Unprecedented Mechanism Employed by the *Salmonella enterica* EutT ATP:Co^{I}rrinoid Adenosyltransferase Precludes Adenosylation of Incomplete Co^{II}rrinoids**

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Abstract: *Three distinct families of ATP:corrinoid adenosyltransferases (ACATs) exist that are capable of converting vitamin B_{12} derivatives into coenzyme B_{12} by catalyzing the thermodynamically challenging reduction of Co^{II}rrinoids to form "supernucleophilic" Co^{I} intermediates. While the structures and mechanisms of two of the ACAT families have been studied extensively, little is known about the EutT enzymes beyond the fact that they exhibit a unique requirement for a divalent metal cofactor for enzymatic activity. In this study we have obtained compelling evidence that EutT converts cob(II)alamin into an effectively four-coordinate Co^{II} species so as to facilitate Co$^{II}\rightarrow$Co^{I} reduction. Intriguingly, EutT fails to promote axial ligand dissociation from the substrate analogue cob(II)inamide, a natural precursor of cob(II)alamin. This unique substrate specificity of EutT has important physiological implications.*

Adenosylcobalamin (AdoCbl), also known as coenzyme B_{12}, is one of the biologically active derivatives of vitamin B_{12}.[1] Homolytic cleavage of the cofactor's Co–C bond represents the first step in the radical-based reactions catalyzed by AdoCbl-dependent enzymes such as ethanolamine ammonia-lyase (EAL),[2] methylmalonyl-CoA mutase (MMCM),[3] and ribonucleotide triphosphate reductase.[4] *Salmonella enterica* sv. Typhimurium LT2 (hereafter *S. enterica*) can synthesize AdoCbl de novo and contains a member of each of the three evolutionarily unrelated families of ATP:corrinoid adenosyltransferases (ACATs), termed CobA, PduO, and EutT.[2,5,6] ACATs catalyze the transfer of the adenosyl (Ado) moiety from a molecule of adenosine triphosphate (ATP) to the Co^{I} center of corrinoid substrates that are either biosynthesized from 5-aminolevulinic acid or salvaged from external sources, such as vitamin B_{12}.[7,8]

The housekeeping ACAT, CobA,[9–13] and the human-type ACAT, PduO,[5,14–17] of *S. enterica* have been the subject of extensive kinetics, structural, and spectroscopic studies, which afforded detailed insight into the mechanism employed by these enzymes to accomplish the thermodynamically challenging Co$^{II}\rightarrow$Co^{I}rrinoid reduction that must precede the adenosylation step. While the reduction potentials of Co^{II}rrinoids in solution (e.g. $E°$(SHE) = −610 mV for cob(II)alamin [Co^{II}Cbl] and −490 mV for cob(II)inamide [Co^{II}Cbi^{+}])[18] are below those of readily available reducing agents, Co^{II}Cbl bound to the PduO-type ACAT from *Lactobacillus reuteri* (*Lr*PduO) and the EutT-type ACAT from *S. enterica* can be reduced by dihydroflavins (e.g. $E°$(SHE) = −228 mV for FMN at pH 7.5).[19,20] Although *Se*CobA-bound Co^{II}rrinoids cannot be reduced by free dihydroflavins, they can be converted into the Co^{I} state by reduced flavoproteins such as flavodoxin.[12,19,21]

The ACAT-bound reducible Co^{II}rrinoid species exhibit several unique spectral features, including an intense, positively signed magnetic circular dichroism (MCD) band in the near-IR region and a series of widely spread resonances in the low-field region of the electron paramagnetic resonance (EPR) spectrum. These features have been attributed to the presence of a significantly stabilized "redox-active" Co $3d_{z^2}$–based molecular orbital (MO), consistent with an effectively four-coordinate, square-planar ligand environment of the Co^{II} center in these species.[13] In support of this proposal, the X-ray crystal structures of substrate-bound *Lr*PduO[15] and *Se*CobA[22] have revealed that a phenylalanine residue at the active site takes up the space typically occupied by the axial ligand of Co^{II}rrinoids, and that the 5,6-dimethylbenzimidazole (DMB) group tethered to the corrin macrocycle of Co^{II}Cbl is excluded from the active site and exposed to solvent.

With regards to EutT, little is known about the structure and function of this enzyme beyond the fact that it requires a divalent metal cofactor (preferentially Fe^{II} or Zn^{II}) coordinated to Cys residues for catalytic activity.[20] To explore if

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[**] This work was supported in part by the National Science Foundation grant MCB-0238530 (to T.C.B.) and the National Institutes of Health grant R37-GM40313 (to J.C.E.-S.). P.E.M was supported in part by Chemical Biology Interface Training Grant T32-GM008505 (L. L. Kiessling, P.I.) from the National Institute of General Medical Sciences (NIGMS).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201501930.

EutT employs a similar mode of substrate Co^{II}rrinoid binding as PduO and CobA, we have carried out MCD and EPR spectroscopic studies of Co^{II}Cbl and $Co^{II}Cbi^+$ in the presence of the *S. enterica* EutT enzyme complexed with Zn^{II} and co-substrate MgATP. As shown in Figure 1, the MCD spectrum

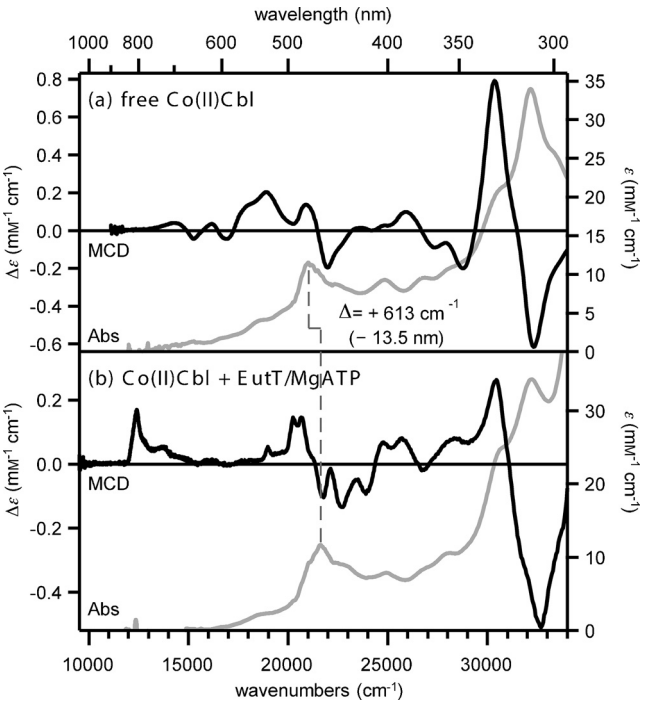

Figure 1. Absorption (gray traces, right axis) and 7 T MCD (black traces, left axis) spectra collected at 4.5 K of a) free Co^{II}Cbl and b) Co^{II}Cbl mixed with EutT that was purified in the presence of excess MgATP under anaerobic conditions (0.4:1.0 ratio). The protein sample was in 0.1 M Tris-HCl buffer (pH 8) containing 0.5 M NaCl and 3 mM MgATP, and both samples contained 60% (v/v) glycerol. The shift of the dominant absorption feature (the α-band) is indicated by the dashed vertical line.

of Co^{II}Cbl changes dramatically in the presence of the EutT/MgATP complex. Most importantly, the prominent positive features appearing at 12410 and approximately 20500 cm^{-1} are highly characteristic of four-coordinate, approximately square-planar Co^{II}rrinoid species.[13,14,17] Binding of Co^{II}Cbl to the EutT/MgATP complex also gives rise to changes in the positions and relative MCD intensities of the corrin $\pi \rightarrow \pi^*$ transitions above 20000 cm^{-1} and to a blue-shift of the major band in the visible region of the electronic absorption spectrum, the so-called α-band (Figure 1). Since the donor molecular orbital involved in the α-band transition contains a sizable contribution from the Co $3d_{z^2}$ orbital,[23] the large blue-shift of this transition in response to Co^{II}Cbl binding to the EutT active site provides further evidence for a significant weakening of the axial bonding interaction.

Consistent with our absorption and MCD data, the EPR spectrum of Co^{II}Cbl in the presence of the EutT/MgATP complex is characteristic of four-coordinate Co^{II}rrinoid species, with resonances spread over an exceptionally broad range of about 4000 G (Figure 2b). The best fit of this spectrum was obtained with a g_3 value of 3.61 and associated

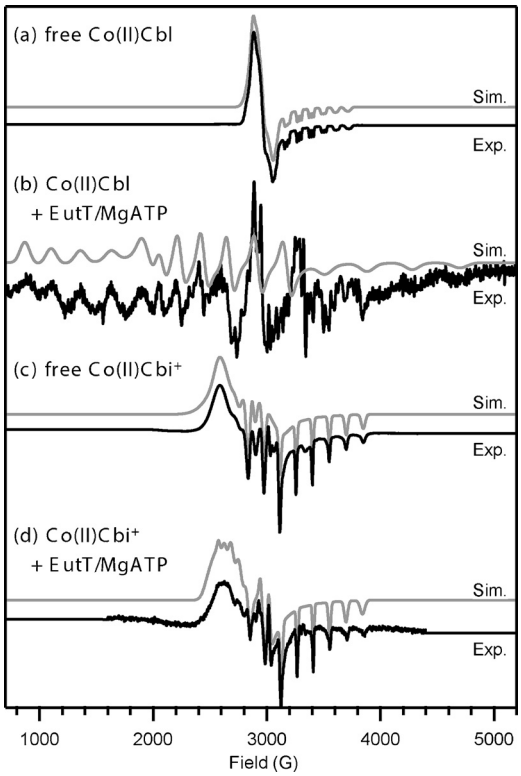

Figure 2. EPR spectra collected at 20 K of a) free Co^{II}Cbl, b) Co^{II}Cbl in the presence of EutT and MgATP, c) free $Co^{II}Cbi^+$, and d) $Co^{II}Cbi^+$ in the presence of EutT and MgATP. The samples were prepared in the same way as those used to obtain the MCD spectra. All spectra were collected with a modulation amplitude of 10 G, a modulation frequency of 100 kHz, and a time constant of 163.84 ms. High-frequency noise in the spectra of the enzyme samples was removed using Fourier transformation. Spectral simulations (gray traces) were performed with the SIMPOW6 program[30] and the fit parameters provided in Table 1.

Co hyperfine coupling constant A_3 of 1362 MHz (Table 1), which are the highest values reported so far for any Co^{II}rrinoid species.[24] Notably, the g_1 value is significantly smaller than 2.0023, which suggests that the unpaired electron no longer resides in a molecular orbital with predominant Co $3d_{z^2}$ orbital character. In support of this assumption, a computational study of Co^{II}Cbl revealed that the Co $3d_{z^2}$- and $3d_{yz}$-based molecular orbitals become nearly degenerate upon removal of the axial ligand.[25]

In contrast to the case of Co^{II}Cbl, the EPR, absorption, and MCD spectra of $Co^{II}Cbi^+$ in the presence of the EutT/MgATP complex do not contain any features attributable to a four-coordinate species (Figure 2d and Figure 3). Nevertheless, small but noticeable differences exist between these spectra and those obtained for free $Co^{II}Cbi^+$, including an

Table 1: EPR g values and ^{59}Co hyperfine values A(Co) (in MHz) used for spectral simulations in Figure 2.

Species	g_1	g_2	g_3	A_1(Co)	A_2(Co)	A_3(Co)
free Co^{II}Cbl	2.001	2.225	2.274	298	33	28
Co^{II}Cbl + EutT/MgATP	1.800	2.553	3.610	760	625	1362
free $Co^{II}Cbi^+$	1.996	2.327	2.441	403	215	249
$Co^{II}Cbi^+$ + EutT/MgATP	1.994	2.312	2.441	393	160	242

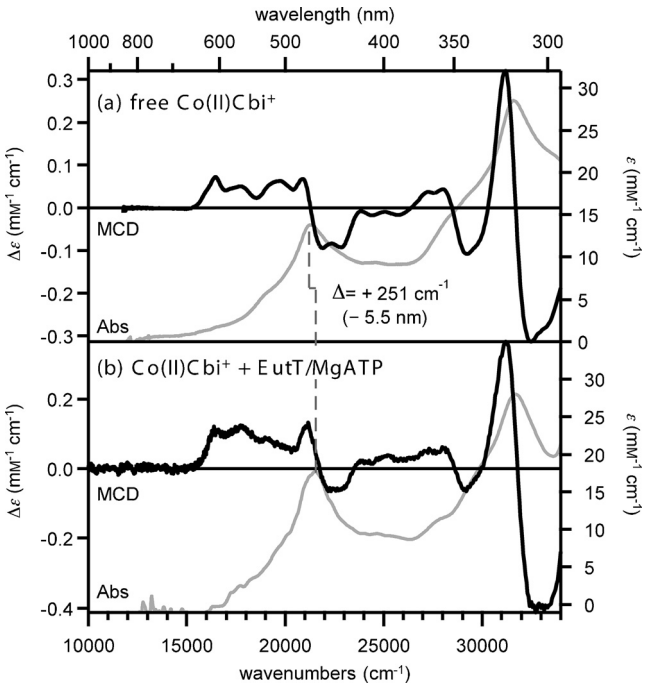

Figure 3. Absorption (gray traces, right axis) and 7 T MCD (black traces, left axis) spectra collected at 4.5 K of a) free $Co^{II}Cbi^+$ and b) $Co^{II}Cbi^+$ in the presence of the EutT/MgATP complex that was prepared as described in the caption of Figure 1 (0.9:1.0 ratio). Both samples contained 60% (v/v) glycerol as a glassing agent. The shift of the dominant absorption feature (the α-band) is indicated by the dashed vertical line.

approximately 250 cm^{-1} blue-shift of the prominent α-band absorption, changes in the relative intensities and positions of the MCD features in the 15000–25000 cm^{-1} range, and a small decrease in the EPR g_2 and A_2 values (Table 1). Hence, it can be concluded that $Co^{II}Cbi^+$ does in fact bind to the enzyme active site, albeit as a five-coordinate species that retains its axial solvent ligand. These findings suggest that the EutT/MgATP complex uses the energy liberated by binding of the nucleotide tail of $Co^{II}Cbl$ to trigger the formation of a four-coordinate species. This mechanism of four-coordinate Co^{II}rrinoid formation is thus fundamentally different from that employed by *Lr*PduO and *Se*CobA, which do not have a specific binding site for the DMB group and are thus capable of adenosylating both $Co^{II}Cbl$ and $Co^{II}Cbi^+$.[13,14]

To corroborate this hypothesis, bioassay and kinetics studies of EutT were performed., Ethanolamine-dependent growth of an *S. enterica* strain unable to express ACAT enzymes (referred to as ΔACAT) was supported by the addition of EutT and $Co^{II}Cbl$, but not by $Co^{II}Cbi^+$ (see Figure S1 in the Supporting Information). While this result was consistent with our observation that EutT is unable to remove the axial solvent ligand from $Co^{II}Cbi^+$ (Figures 2 and 3), it could have also indicated that cobinamides cannot actually bind to the active site of the enzyme. Therefore, an additional bioassay was performed in which we monitored the ethanolamine-dependent growth of the ΔACAT strain in the presence of EutT and $Co^{I}Cbl^-$ or $Co^{I}Cbi$, which lack any axial ligands. The results from this experiment (see Figure S2 in the Supporting Information) provided conclusive evidence that EutT can adenosylate complete and incomplete Co^{I}rrinoids. Next, to verify whether $Co^{I}Cbl^-$ and $Co^{I}Cbi$ compete for the same substrate-binding site of EutT, we performed a competition experiment in which the rate of adenosylation of $Co^{I}Cbl^-$ by EutT/MgATP was mesured in the presence of various amounts of $Co^{I}Cbi$. As shown in Figure 4, a negative relationship exists between the rate of $Co^{I}Cbl^-$ adenosylation and the concentration of $Co^{I}Cbi$ used, consistent with the latter being a competitive subtrate inhibitor. The inhibitory effect of $Co^{I}Cbi$ is relatively small, which is suggestive of a weak binding of this alternative substrate to the EutTMgATP active site. Collectively, our bioassay and kinetics experiments confirm that EutT fails to accomplish the $Co^{II}\rightarrow Co^{I}$rrinoid reduction under physiologically relevant conditions and corroborate our hypothesis that the enzyme 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. Although in the absence of X-ray crystallogaphic data it is not possible to identify the amino acid residues that make up the DMB binding site, it is interesting to note that in MMCM[26] and glutamate mutase,[27] two AdoCbl-dependent enzymes that bind the cofactor in the "His-on" form, the nucleotide loop runs through a cavity with well-ordered water molecules and the DMB base is deeply buried in a hydrophobic pocket. In both enzymes, a serine residue forms a hydrogen bond to the nitrogen atom of the DMB base that coordinates to the cobalt ion in free $Co^{II}Cbl$.

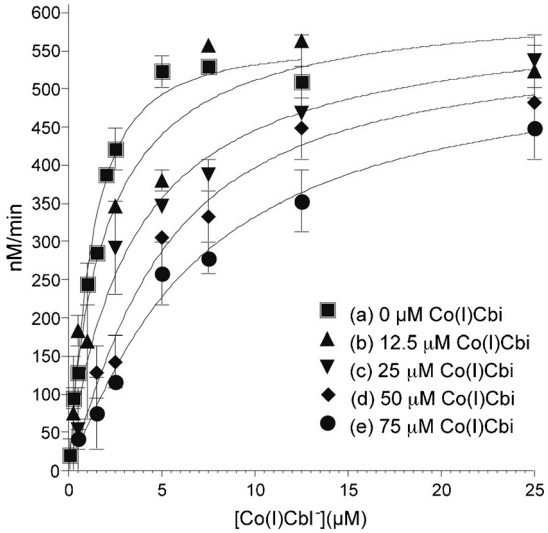

Figure 4. Competitive inhibition of EutT by $Co^{I}Cbi$ in a continuous spectrophotometric assay that monitors product AdoCbl formation at 525 nm. The concentration of $Co^{I}Cbl^-$ was held constant at several values (from 0–25 μM) as the concentration of $Co^{I}Cbi$ was varied. K_M values (μM) are as follows: a) 1.2 ± 0.2; b) 2.1 ± 0.3; c) 3.4 ± 0.8; d) 4.7 ± 1.2; e) 6.6 ± 1.4. V_{max} values (nM min^{-1}) are as follows: a) 559 ± 31, b) 633 ± 94, c) 600 ± 105, d) 558 ± 84, e) 541 ± 101.

In conclusion, despite the fact that EutT is both structurally and evolutionarily unrelated to the CobA and PduO families of ACATs, it uses the same general strategy to overcome the thermodynamic barrier associated with the

$Co^{II} \rightarrow Co^{I}$ reduction, converting the five-coordinate Co^{II}Cbl substrate into a four-coordinate species. However, unlike the other ACATs which accomplish this task by displacing the axial ligand with a hydrophobic phenylalanine residue, EutT appears to employ a mechanism for Co–N(DMB) bond dissociation that involves binding of the nucleotide loop of Co^{II}Cbl to a site remote from the Co^{II} center. As a result, EutT fails to convert $Co^{II}Cbi^{+}$ into a four-coordinate species, even though the Co–O(water) bond is weaker than the Co–N(DMB) bond of Co^{II}Cbl. Intriguingly, a similar prerequisite for the presence of the nucleotide tail has been documented for MMCM, which binds AdoCbl in the "base-off/His-on" form but fails to induce a ligand switch when the native cofactor is replaced by $AdoCbi^{+}$.[28] The substrate specificity of EutT may suggest that the AdoCbl product is not released into solution but rather transferred directly to the acceptor enzyme, EAL, as has been proposed previously for AdoCbl transfer from hATR to MMCM.[29] By selectively adenosylating Co^{II}Cbl, EutT could prevent the incorporation of $AdoCbi^{+}$ into the EAL active site, thereby precluding the formation of inactive enzyme.

Keywords: adenosylcobalamin · adenosyltransferases · enzyme catalysis · reaction mechanisms · reduction

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 7158–7161
Angew. Chem. **2015**, *127*, 7264–7267

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Received: February 28, 2015
Published online: April 27, 2015

