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Multiple Roles of ATP:Cob(I)alamin Adenosyltransferases in the Conversion of B₁₂ to Coenzyme B₁₂

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Abstract

Our mechanistic understanding of the conversion of vitamin B₁₂ into coenzyme B₁₂ (a.k.a. adenosylcobalamin, AdoCbl) has been substantially advanced in recent years. Insights into the multiple roles played by ATP:Cob(I)alamin adenosyltransferase (ACA) enzymes have emerged through the crystallographic, spectroscopic, biochemical, and mutational analyses of wild-type and variant proteins. ACA enzymes circumvent the thermodynamic barrier posed by the very low redox potential associated with the reduction of cob(II)alamin to cob(I)alamin by generating a unique four-coordinate cob(II)alamin intermediate that is readily converted to cob(I)alamin by physiological reductants. ACA enzymes not only synthesize AdoCbl, they deliver it to the enzymes that use it, and, in some cases, enzymes whose function is needed to maintain the fidelity of the AdoCbl delivery process have been identified. Advances in our understanding of ACA enzyme function have provided valuable insights into the role of specific residues, and into why substitutions of these residues have profound negative effects on human health. From an applied science standpoint, a better understanding of the adenosylation reaction may lead to more efficient ways of synthesizing AdoCbl.

Keywords

Coenzyme B₁₂; Adenosylcobalamin; Corrinoid adenosylation; Cyclic tetrapyrroles; Vitamin metabolism

General features of adenosylcobalamin

Coenzyme B₁₂ (5'-deoxyadenosylcobalamin, AdoCbl) is a structurally complex molecule that is essential to animals, lower eukaryotes, and many prokaryotes (Escalante-Semerena & Warren 2008). This unique coenzyme is involved in catabolic and anabolic processes, including DNA synthesis. In mammals, AdoCbl is involved in the catabolism of amino acids, cholesterol and odd-chain fatty acids (Banerjee & Vlasie 2002; Banerjee & Chowdhury 1999; Rosenblatt & Fenton 1999). In humans, the inability to generate AdoCbl leads to methylmalonic aciduria, a fatal disorder associated with developmental retardation and infant mortality (Dobson *et al.* 2002; Fenton & Rosenberg 1978; Ciani *et al.* 2000; Cavicchi *et al.* 2006).

AdoCbl is one of the most complex coenzymes in nature (Fig. 1), and belongs to the family of cyclic tetrapyrroles, which include heme, chlorophyll, and factor F₄₃₀ (Battersby 2000; Montforts & Glasenapp-Breiling 2002). In AdoCbl, the cobalt ion is held in place by four equatorial nitrogen ligands from the pyrrole moieties. This Co-containing cyclic

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tetrapyrrole ring is known as the corrin ring. In nature, corrin rings are found with a variety of upper (*Coβ*) and lower (*Coα*) axial ligands. In the case of AdoCbl, the upper ligand is a 5'-deoxyadenosyl (Ado) moiety, and the lower ligand is a nitrogen atom from 5,6-dimethylbenzimidazole (DMB) (Lenhart 1968). The lower ligand base is tethered to the corrin ring via a structure known as the nucleotide loop, which is unique in several respects. Firstly, the *N*-glycosidic bond between DMB and the ribose is in the α configuration, rather than the β configuration usually found in nucleotides. Secondly, the phosphate group of the nucleotide is attached to the 3' hydroxyl rather than the usual 5' hydroxyl group of the ribose moiety. And thirdly, AdoCbl is one of only three coenzymes known to contain a phosphodiester bond; the other two are coenzyme F₄₂₀ (Eirich *et al.* 1978), and methanopterin (van Beelen *et al.* 1984). AdoCbl-dependent enzymes bind the coenzyme in two different conformations known as the base-on (DMB-on) and base-off (DMB-off) conformation (Fig. 1).

The lability of the organometallic (Co-C) bond between the Co ion and the Ado upper ligand lies at the heart of the reactivity of AdoCbl (Fig. 1). This Co-C bond is unique in nature because unlike other organometallic bonds it is relatively stable, but easy to break. The properties of this Co-C bond allows AdoCbl to facilitate intramolecular rearrangements (Banerjee & Chowdhury 1999; Buckel *et al.* 1999), deaminations (Bandarian & Reed 1999), dehydrations (Toraya 1999), reductions (Fontecave 1998; Fontecave & Mulliez 1999), and reductive dehalogenations (Wohlfarth & Diekert 1999) via radical intermediates.

Formation of the Co-C bond; the corrinoid adenosylation pathway

AdoCbl is the coenzymic form of vitamin B₁₂ (cyanocobalamin, CNCbl). The cyano group of vitamin B₁₂ is the result of the method used for the isolation of Cbl (Veer *et al.* 1950; Wijmenga *et al.* 1950; Spalla *et al.* 1989).

In humans, vitamin B₁₂ is absorbed from our diet and transported by proteins with exceptionally high affinities for this vitamin. Once inside the cell, Cbl is activated into AdoCbl in the mitochondrion, or remains in the cytosol, where it participates as a methyl carrier in the conversion of homocysteine to methionine (Banerjee 2006).

The complete *in vitro* synthesis of vitamin B₁₂ (>70 chemical reactions) was the culmination of a 12-year collaborative effort between the groups of R. B. Woodward (MIT, Cambridge, USA) and A. Eschenmoser (ETH, Zürich, Switzerland) (Woodward 1973; Eschenmoser & Wintner 1977). Although bacteria and archaea have trimmed the number of reactions needed to synthesize AdoCbl, they still dedicate a great deal of genetic information to the assembly of this complex molecule (Escalante-Semerena & Warren 2008).

The activation of vitamin B₁₂ to AdoCbl involves the transfer of the adenosyl (Ado) moiety from ATP to the reduced Co^{I+} ion of cobalamin [cob(I)alamin]. This activation is catalyzed by ATP:Cob(I)alamin adenosyltransferase (ACA) enzymes. In this reaction, the Co^{I+} nucleophile attacks the 5'C of ATP generating the unique cobalt-carbon bond of AdoCbl. Recent detailed structural and functional characterization of these enzymes led to the discovery that ACA enzymes are multifunctional, since they also facilitate the generation of the corrinoid substrate, and play a role in the delivery of AdoCbl to the enzymes that use it. In this review, we discuss recently obtained insights into the mechanism of function by ACA enzymes.

Distribution of ACA enzymes

Cells from all domains of life synthesize ACA enzymes, which are of three types CobA, PduO, EutT. Surprisingly, CobA, EutT and PduO do not share sequence similarity at the

nucleotide, amino acid, or tertiary structural level, suggesting separate lines of evolution. The Gram-negative enterobacterium *Salmonella enterica* synthesizes all three types (Suh & Escalante-Semerena 1995; Johnson *et al.* 2001; Buan & Escalante-Semerena 2006; Johnson *et al.* 2004), yet this bacterium uses each enzyme under different physiological conditions (Escalante-Semerena *et al.* 1990; Johnson *et al.* 2001; Buan *et al.* 2004; Sheppard *et al.* 2004).

The first ACA enzyme characterized was *SeCobA*, which is regarded as the housekeeping ACA enzyme in *S. enterica* (Escalante-Semerena & Warren 2008). *SeCobA* is involved in the *de novo* synthesis of Cbl, and in the scavenging of incomplete corrinoids (Escalante-Semerena *et al.* 1990). The activity of *SeCobA* is needed because several enzymes involved in the late steps AdoCbl biosynthesis require adenosylated corrinoid substrates (Escalante-Semerena & Warren 2008). Spectroscopic evidence suggests that *SeCobA* adenosylates incomplete corrinoids more efficiently than Cbl (Stich *et al.* 2005a).

S. enterica uses PduO- and EutT-type ACA enzymes for the catabolism of 1,2-propanediol and ethanolamine, respectively. The PduO-type is the most widely distributed ACA enzyme, and is the type of enzyme found in human cells (Leal *et al.* 2003). In humans, adenosylation of Cbl takes place in the mitochondrion (Banerjee & Chowdhury 1999). From a mechanistic standpoint, PduO-type ACA enzymes are best understood. In contrast, little is known about EutT. Initial biochemical characterization of EutT suggests that this type of ACA enzyme requires a metal ion for activity (Buan *et al.* 2004).

Structural characterization of ACA enzymes

Crystal structures of the CobA and PduO-type ACA enzymes have been solved at high resolution. Due to the difficulty purifying EutT, a model for the structure of this protein was not available at the time of this writing.

Structural characterizations of *SeCobA*, and several PduO-type enzymes from different sources, revealed that although these two enzymes catalyze the same reaction, they do not share tertiary nor quaternary structure homology (Fig. 2). Moreover, the *SeCobA*, the *Lactobacillus reuteri* (*LrPduO*), and the human adenosyltransferase (hATR) bind substrates in their active sites in different conformations (Bauer *et al.* 2001; St Maurice *et al.* 2008; St Maurice *et al.* 2007; Schubert & Hill 2006).

The *SeCobA* enzyme is a homodimer consisting of α/β structure with topology belonging to the P-loop containing family of nucleotide hydrolases (Bauer *et al.* 2001). Notably, the P-loop of CobA is one amino acid shorter than the consensus motif. In *SeCobA*, ATP is bound to the P-loop in a conformation that exposes the 5'C of ATP to the Co^{1+} nucleophile (Bauer *et al.* 2001). Such an orientation favors the transfer of the Ado moiety, rather than the γ phosphate group. In the crystal structure available in the database (PDB code 1G64), Cbl is bound to the active site with the lower ligand coordinated to the Co ion, a conformation known as the base-on conformation (Fig. 2). There are several unresolved questions regarding the *SeCobA* structure. First, the distance between the Co ion and the 5'C of ATP is too long for the nucleophilic attack to occur. Second, spectroscopic analyses revealed that adenosylation takes place via a four-coordinate cob(II)alamin DMB-off conformation intermediate (Stich *et al.* 2005a; Stich *et al.* 2004), however, as noted above, Cbl is bound in the active site in the base-on conformation, a five-coordinate species. A conformational change of CobA where the enzyme displaces the lower ligand of Cbl, and brings the Co ion in close proximity to the 5'C for the nucleophilic attack to take place would help explain these discrepancies.

In recent years, several groups reported structural characterizations of PduO-type ACA enzymes from several sources. An archaeal PduO-type enzyme with no substrates in the active site was reported to form a trimer with each subunit composed of five-helix bundles (Saridakis *et al.* 2004). The structural analysis of a bacterial and the human PduO-type enzymes showed that the active site of this class of ACA enzyme is formed by the *N*-terminus of one subunit and the *C*-terminus of the adjacent subunit (Schubert & Hill 2006; St Maurice *et al.* 2007). The structures of the proteins in complex with ATP uncovered a novel ATP binding motif (Thr-(Lys/Arg)-X-Gly-Asp-X-Gly-X-(Thr/Ser) that is unique to PduO-type ACA enzymes. The structure of *Lr*PduO/ATP/Cbl ternary complex provided visual evidence of the existence of a base-off, four-coordinate cob(II)alamin intermediate (St Maurice *et al.* 2008) (Fig. 2).

How PduO-type ACA enzymes generate the Co¹⁺ nucleophile

As mentioned above, the reduced Co¹⁺ ion of cob(I)alamin is the nucleophile that attacks the 5'C of ATP (Fig. 3). Therefore the substrate of ACA enzymes is cob(I)alamin, a molecule with two important features. First, cob(I)alamin is very reactive, and, if left unprotected, it is rapidly oxidized to Co²⁺. Second, the midpoint redox potential of the Co²⁺/Co¹⁺ couple [$E^{\circ} = -610$ mV, (Lexa & Saveant 1976)] is too low for any known cellular reductant, thus the generation of cob(I)alamin is a challenging, thermodynamically unfavorable reaction. ACA enzymes solve both problems by preferentially binding Co²⁺ corrinoids, and generating a four-coordinate species that can be reduced by free or protein bound dihydroflavins (Mera & Escalante-Semerena 2010) (Fig. 3).

Elegant spectroscopic studies by T. Brunold and co-workers provided insights into the strategy used by *Se*CobA and *Lr*PduO/hATR to overcome the thermodynamic barrier posed by the reduction of cob(II)alamin (Stich *et al.* 2005a; Stich *et al.* 2005b; Park *et al.* 2008). To solve this problem, ACA enzymes raise the reduction potential of the Co²⁺ ion to within physiological range by displacing the lower ligand of Cbl, generating a four-coordinate cob(II)alamin intermediate. Detailed structural and kinetic analyses of *Lr*PduO revealed the critical role of a conserved bulky residue (phenylalanine, F112 in *Lr*PduO), which displaces the lower ligand (Mera *et al.* 2009).

The source of the electron used to reduce the ACA enzyme-bound cob(II)alamin to cob(I)alamin remains an unresolved question. Although several putative electron sources have been suggested for CobA- and PduO-type enzymes, there is no *in vivo* evidence in support of these ideas. *In vitro*, *Se*CobA was shown to interact with flavodoxin A (FldA), a well-studied flavin mononucleotide (FMN)-dependent electron transfer protein, whose interactions with the Cbl-dependent methionine synthase (MetH) enzyme of *E. coli* have been elegantly documented by Rowena Matthews and co-workers (Hoover *et al.* 1997; Hall *et al.* 2000; Hall *et al.* 2001). Reduced FldA was shown to efficiently drive the adenosylation of cob(II)alamin (Fonseca & Escalante-Semerena 2001; Buan & Escalante-Semerena 2005). In *Brucella melitensis* and in *S. enterica*, the CobR and PduS flavoproteins, were respectively shown *in vitro* to reduce cob(III)alamin in two consecutive one-electron reductions to cob(I)alamin (Lawrence *et al.* 2008; Sampson *et al.* 2005). Recently, we found that free reduced flavins, and non-specific flavoproteins can reduce cob(II)alamin bound in the active site of ACA enzymes (Mera & Escalante-Semerena 2010). These results prompted us to consider the questions of whether there is the need of a protein involved in this reduction, and if there is, is such a protein an electron transfer protein, or an enzyme. Based on data available at this point, we have suggested that bona fide cobalamin reductases have not been identified (Mera & Escalante-Semerena 2010).

Additional details about the adenosylation reaction

ACA enzymes follow an ordered sequential binding scheme, where ATP binds first (Stich et al. 2005a; Mera et al. 2007; St Maurice et al. 2007; Suh & Escalante-Semerena 1995). ACA enzymes can bind and transfer the nitrogenous base from different nucleoside triphosphates to cob(I)alamin, albeit not with the same efficiency (Fonseca et al. 2002; Johnson et al. 2004; St Maurice et al. 2007; Mera et al. 2007; Buan & Escalante-Semerena 2006). In the case of *LrPduO*, two functional groups of ATP explain the reduced ability of the enzyme to use alternate nucleotides, i) the amino group at the C-6 position, which contributes to the affinity of the enzyme, and ii) the nitrogen at the N-7 position, which is involved in catalysis (Mera et al. 2007). Unlike *PduO* and *EutT*, *SeCobA* requires the 2'OH group of the ribosyl moiety of ATP for activity (Fonseca et al. 2002).

Unlike most of ATP-dependent enzymes, *PduO* and *CobA* eliminate the tripolyphosphate group of ATP, whereas P_i and PP_i were shown to be the byproducts of the *EutT* reaction (Fonseca et al. 2002; Johnson et al. 2004; St Maurice et al. 2007; Buan & Escalante-Semerena 2006).

Critical residues of the *PduO*-type ACA enzymes have been studied more closely, specifically because mutations in the human adenosyltransferase (hATR) have been found in patients with methylmalonic aciduria (Zhang et al. 2006). Detailed genetic, structural, and kinetic analyses have unveiled why these substitutions are deleterious to the function of the enzyme. One key residue that has been characterized by several groups is the conserved arginine 186 (position in hATR), whose change to a tryptophan is the most common substitution found in hATR (Lerner-Ellis et al. 2006). Arg186 is critical for catalysis, even though it does not directly interact with ATP (Schubert & Hill 2006; St Maurice et al. 2007; Mera et al. 2007; Fan & Bobik 2008; Zhang et al. 2009). From studies performed with the bacterial *LrPduO*, we now understand that Arginine 186 (Arg128 in *LrPduO*) forms a salt bridge with a conserved aspartate from the adjacent subunit (St Maurice et al. 2007; Mera et al. 2007). Structural and kinetic analyses of *LrPduO* revealed that the salt bridge affects the rate at which the enzyme/ATP complex forms. In other words, changes in the ability to form this salt bridge affects the catalytic efficiency of the enzyme (Mera et al. 2007). Furthermore, the structure of *LrPduO*/ATP/Cbl complex unveiled a direct contact between the side chain Arg186 (Arg128 in *LrPduO*) and one of the corrin ring amide (St Maurice et al. 2008). In *LrPduO*, a conserved substitution R128K resulted in a 9-fold drop in the affinity of the enzyme for cob(I)alamin. Intriguingly, the mutation of this residue to a tryptophan in hATR was reported by Gravel and co-workers to have a defect in binding AdoCbl but not a defect in binding cob(I)alamin. Additional kinetic and structural analyses of this tryptophan variant in *LrPduO* or hATR are needed to clearly understand the function of the interaction between this conserved arginine residue and the corrin ring. Certainly, affinity is not the only problem generated by the change at that position since a hATR^{R186W} variant is inactive (Saridakis et al. 2004; Zhang et al. 2006).

After facilitating the generation of substrate and catalyzing the formation of product, ACA enzymes ensure that this valuable coenzyme gets to the right target enzyme (Fig. 3). The concentrations of AdoCbl inside the cell are very low making simple diffusion the unlikely mechanism by which AdoCbl-dependent enzymes get AdoCbl (Banerjee 2006). In humans, methylmalonyl-CoA mutase (MCM) is the AdoCbl-dependent enzyme that isomerizes methylmalonyl-CoA into succinyl-CoA inside the mitochondrion (Mancia et al. 1996). Ruma Banerjee and co-workers have shown that the *PduO* enzyme from *M. extorquens* (*MePduO*) transfers AdoCbl directly to the methylmalonyl-CoA mutase (MCM) (Padovani et al. 2008). MCM binds AdoCbl in the base-off/His-on conformation, and it is this histidine residue that plays a critical role in the transfer of the coenzyme from *MePduO* to MCM (Padovani et al. 2008; Mancia et al. 1996). Results from kinetic and thermodynamic analyses

of the *MePduO* enzyme support a rotary mechanism in which, at any given time, only two of its active sites are used for AdoCbl synthesis (Padovani & Banerjee 2009b). In this proposed mechanism, the binding of ATP to the vacant active site of PduO induces the release/transfer of product to the acceptor enzyme.

In addition, the same group has assigned the GTPase chaperone protein MeaB a key role in the fidelity of the AdoCbl loading process (Padovani & Banerjee 2009a). The data obtained by these authors is supported by the finding of a substitution in the human orthologue of MeaB in a patient with methylmalonic aciduria; the mutant MeaB fails to discriminate between active and non-active cofactors (Padovani & Banerjee 2009a).

A role for ACA enzymes in the delivery of cancer-fighting drugs

In the fight against cancer, researchers would like to advantage of the fact that Cbl is an essential nutrient of human cells, and that tumor cells have a higher requirement for Cbl. The idea of using Cbl as a means to deliver tumor-fighting drugs to a target has been explored in the past (Bagnato *et al.* 2004), and a recent report showed that ACA enzymes efficiently use cisplatin-like conjugates of Cbl as substrates, with the concomitant production of AdoCbl, and the release of the drug (Ruiz-Sanchez *et al.* 2008). The use of Cbl conjugates to fight cancer seems promising.

AdoCbl production

The structural complexity of AdoCbl makes the chemical synthesis of this molecule too expensive for industrial production. In fact, the need to mass produce vitamin B₁₂ drove the isolation of proteins involved in the biosynthesis of Cbl by bacteria (Warren *et al.* 2002). At present, more than 80% of the world's production of vitamin B₁₂ is synthesized by the fermentation using genetically engineered *Pseudomonas dentrificans* strains (Martens *et al.* 2002).

Bacteria efficiently synthesize AdoCbl, however, the sensitivity of the cobalt-carbon bond of AdoCbl to light, heat, and reagents such as cyanide, complicates its purification. Stable, and readily purifiable ACA enzymes offer a feasible alternative to AdoCbl production. Recent advances in our understanding of how the cob(I)alamin substrate is generated, further make the use of ACA enzymes for AdoCbl production a viable alternative.

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Fig. 1. Schematic representation of adenosylcobalamin (AdoCbl). The adenosyl moiety of AdoCbl is shown in red. The corrin ring, nucleotide loop, and the lower ligand DMB is known as Cbl

Fig. 2.

X-ray crystallography models of CobA and PduO ACA enzymes. **a.** The dimer of *SeCobA* is shown using ribbon representation with each subunit colored in cyan and gold; **b.** The trimer of *LrPduO* is shown with each subunit colored yellow, violet and green. **c.** *SeCobA*'s active site. The location and conformation of ATP (black) and Cbl (pink) are shown using the stick format. Cbl is bound in the active site of CobA in the DMB-on conformation. **d.** *LrPduO*'s active site. The substrates ATP (black) and Cbl (pink) are shown in the active site with Cbl bound in the DMB-off conformation. For simplicity, only one active site of CobA and PduO are shown with substrates bound. Figure was generated using the PyMOL program (<http://www.pymol.org>).

Fig. 3.

Schematic mechanism of the multiple functions performed by ACA enzymes. ACA enzymes (green oval) facilitate the reduction to cob(I)alamin by binding cob(II)alamin (rectangle) and generating a four-coordinate cob(II)alamin intermediate. After catalysis, the product AdoCbl is delivered to the AdoCbl-dependent enzyme (violet open-squares), a process that is facilitated by a GTPase chaperone.