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The tinker, tailor, soldier in intracellular B₁₂ trafficking

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Abstract

The recognition of eight discrete genetic complementation groups among patients with inherited cobalamin disorders provided early insights into the complexity of a cofactor-processing pathway that supports only two known B₁₂-dependent enzymes in mammals. With the identification of all eight genes now completed, biochemical interrogations of their functions have started and are providing novel insights into a trafficking pathway involving porters that tinker with and tailor the active cofactor forms and editors that ensure the fidelity of the cofactor loading process. The principles of sequestration and escorted delivery of a rare and reactive organometallic cofactor that are emerging from studies on B₁₂ might be of general relevance to other cofactor trafficking pathways.

Cobalamins or derivatives of vitamin B₁₂ are complex organometallic cofactors (Figure 1) that are utilized by a limited selection of enzymes, which typically catalyze chemically challenging reactions exploiting the reactivity of the cobalt–carbon bond. Although required by only two mammalian enzymes: the methylcobalamin (MeCbl)-dependent methionine synthase (MS) and the 5′-deoxyadenosylcobalamin (AdoCbl)-dependent methylmalonyl-CoA mutase (MCM), B₁₂ is an essential micronutrient, whose complete absence is incompatible with life. Unlike some bacteria that can synthesize this complex cofactor *de novo*, mammals obtain it from the diet. Brilliant clinical studies on patients with inborn errors of cobalamin metabolism had led to their distinction into eight genetic complementation groups, *cbIA-G* and *mut* [1–3], and provided early insights into a complex pathway for intracellular processing of B₁₂. To date, all eight genes responsible for the defects in intracellular cobalamin metabolism have been identified but their functions await elucidation.

Given the relative rarity of the cofactor (30–700 nM in humans [4]) and its reactivity in all three biologically relevant oxidation states, we have posited that cobalamin is escorted on its intracellular journey from its point of entry into the lysosome to its destinations in the cytoplasm and mitochondrion, respectively (Figure 2) [5*]. The problem with cofactor delivery from a limited free intracellular pool is compounded by it being predominantly in the ‘base-on’ conformation in solution whereas it is in the ‘base-off’ state in the mammalian B₁₂-dependent enzymes (Figure 1). The base refers to the endogenous 5,6-dimethylbenzimidazole group that is tethered by a spacer to the tetrapyrrole ring and serves

as the lower axial ligand to the cobalt. Protonation of the base, governed by a pK_a of 2.9 and 3.7 for MeCbl and AdoCbl, respectively [6], leads to the ‘base-off’ state, which is present at vanishingly low concentrations at physiological pH. These factors combine to make collision-based delivery of B₁₂ to its target enzymes a considerable challenge. In this review, we focus on the recent advancements in our understanding of cellular strategies for sequestration and delivery of cobalamin and the proteins that tinker with and tailor the active cofactors and guard the fidelity of the cofactor docking process.

The early steps in cobalamin import and processing

Absorption, transport, and cellular uptake of cobalamin

Vitamin B₁₂ derived from the diet embarks on a carrier-mediated import system by binding first to the salivary glycoprotein, haptocorrin, which exhibits a very high affinity for its B₁₂ cargo [7] especially under acidic conditions (pH <3), and protects cobalamin from hydrolysis. It has a broad specificity for B₁₂ and also binds analogs, for example cobamides in which the nucleotide tail is missing [8,9]. Haptocorrin escorts cobalamin through the stomach to the duodenum where pancreatic proteases release the vitamin for loading onto a second glycoprotein binder, intrinsic factor (IF). Pernicious anemia results from IF deficiency. Unlike haptocorrin, IF specifically binds intact B₁₂ derivatives containing the nucleotide tail and serves as the first checkpoint for quality control in the cofactor assimilation pathway. The intestinal absorption of IF·B₁₂ by mucosal cells occurs via cubilin-receptor-mediated endocytosis at the apical side of the brush-border membranes [10]. Subsequently, IF is degraded and B₁₂ appears in the blood stream where it associates with transcobalamin II (TCII). Haptocorrin is also found in circulation and ~80% of the cobalamin is bound to this protein [11]. TCII binds cobalamin in the ‘base-on’ conformation [12] with femtomolar affinity [7], and only binds the intact cofactor, thus representing the second level of scrutiny for distinguishing intact from degraded cofactor. Only the TCII·B₁₂ complex is internalized by a specific transcobalamin receptor (TCR) whereas holo-haptocorrin appears to serve as a B₁₂ reservoir that can be mobilized under conditions of deficiency.

The gene encoding TCR has been identified recently [13*]. The TCR:TCII·B₁₂ complex is internalized into the lysosome where TCII is degraded releasing B₁₂, while the TCR is recycled (Figure 2). We have speculated that transit through the acidic milieu of the lysosome increases the concentration of the ‘base-off’ form of B₁₂ and that once the cofactor exits this compartment it remains both ‘base-off’ and protein-bound [5*].

Lysosomal export mediated by LMBD1, a putative membrane protein

Egress of cobalamin from the lysosome requires a specific transporter whose functional deficiency leads to the lysosomal accumulation of B₁₂ and is a hallmark of the *cbF* group of cobalamin disorders [14]. The genetic locus responsible for the *cbF* defect has been recently identified as the *LMBRD1* gene encoding a membrane protein, LMBD1, related to the lipocalin-1 interacting membrane receptor, LMR [15**]. Genetic defects in the *LMBRD1* gene affect both the cytosolic and mitochondrial branches of cobalamin metabolism. LMR internalizes cargo bound to tear lipocalin [16] and to β-lactoglobulin [17], which binds the

tetrapyrrole, protoporphyrin IX. This leads to the hypothesis that an additional protein might be required to bind free lysosomal B₁₂ for delivery to the exporter, LMBD1 [15**].

MMACHC, the missing decyanase and dealkylase

Mutations in the *MMACHC* (for *methylmalonic aciduria type C* and *homocystinuria*) gene are responsible for the *cbC* class of cobalamin disorders [18**]. The C-terminal domain of the encoded protein is homologous to TonB, which is involved in the transport of cargo across the outer membrane in Gram-negative bacteria. On the basis of this resemblance, the N-terminal domain of MMACHC is predicted to interact with the lysosomal LMBD1 transporter, allowing passage of cobalamin into the cytoplasm [5*,18**].

Despite its unconventional B₁₂-binding motif [18**], human MMACHC binds B₁₂ stoichiometrically showing relaxed specificity for the upper axial ligand, consistent with its postulated role in accepting diverse B₁₂ derivatives (with intact dimethylbenzimidazole tails) entering the cytoplasm. MMACHC binds alkylcobalamins in the ‘base-off’ mode [19**]. However, MMACHC functions as more than a porter catalyzing removal of the upper axial ligands from bound B₁₂ derivatives yielding a cobalamin intermediate, which can be subsequently converted to the active MeCbl and AdoCbl forms. With CNCbl, MMACHC catalyzes a reductive decyanation reaction using reducing equivalents transferred from NADPH via a flavoprotein oxidoreductase [19**]. The product of the decyanation reaction, cob(II)alamin, is bound in the ‘base-off’ state in the active site of MMACHC. This conformation may be necessary for its subsequent delivery to the target enzymes that elaborate MeCbl and AdoCbl in the cytoplasm and mitochondrion, respectively. The ability of MMACHC to decyanate CNCbl explains the clinical observations that *cbC* patients respond to treatment with aquocobalamin but poorly to CNCbl [20].

What is the fate of alkylcobalamins entering the cytoplasm? In the event that the biologically relevant AdoCbl and MeCbl forms are transferred to MMACHC, they are likely to undergo dealkylation to yield a product that can be subsequently partitioned for MeCbl and AdoCbl synthesis. Cell culture studies with fibroblasts exposed to various [⁵⁷Co]-alkylcobalamins reveal that they are converted to the biologically relevant forms, AdoCbl and MeCbl, upon cellular uptake, a process that is impaired in *cbC* cell lines [21]. These results are consistent with a role for MMACHC in the dealkylation of alkylcobalamins. The dealkylation mechanism clearly must be distinct from the reductive decyanation reaction. In summary, MMACHC is a versatile protein, a porter that is postulated to open the hatch on a lysosomal transporter to receive the B₁₂ cargo, a decyanase, and a dealkylase.

MMADHC: a transporter or an adaptor protein?

MMADHC (for *methylmalonic aciduria type D* and *homocystinuria*) has been recently identified as the gene corresponding to the *cbD* locus [22**]. The particularity of this complementation group is that patients with defects in MMADHC present clinical heterogeneity that range from isolated homocystinuria or methylmalonic aciduria to combined methylmalonic aciduria and homocystinuria [23,24]. Analysis of the *MMADHC* gene in *cbD* patients with isolated methylmalonic aciduria reveals that the mutations are clustered in the N-terminal region of the encoded protein whereas they map to the C-

terminal region in patients with isolated homocystinuria, suggesting the existence of two functional domains [22**]. Alternatively, the heterogeneity observed with these patients might be explained by isoforms of the protein resulting from alternative translation start sites on the same message. Hence, mutations in the N-terminal region might disrupt the predicted mitochondrial targeting sequence leading to impaired AdoCbl synthesis whereas mutations in the C-terminal region might disrupt interactions with MS leading to impaired MeCbl synthesis. Although the clinical data provide strong evidence for the dual role of this protein in supporting the cytoplasmic and the mitochondrial pathways for B₁₂ assimilation, biochemical insights into the function of this protein in intracellular B₁₂ trafficking are lacking. It is unclear whether MMADHC binds B₁₂ (it has a weakly conserved B₁₂-binding motif) or whether it functions as an adaptor for the complexation of MMACHC-cob(II)alamin to acceptor proteins in the cytoplasm (MS) and in the mitochondrion (presumably a transporter) (Figure 2). MMADHC belongs to an uncharacterized family of proteins with sequence similarity over a stretch of 91 amino acids to the ATPase component of a putative ABC transporter [22**]. However, MMADHC is missing the Walker A, Walker B, and the ABC signature motifs characteristic of all classic ABC transporters.

The methylcobalamin branch

The *cbfG* locus encodes MS, which catalyzes the MeCbl-dependent conversion of homocysteine to methionine [25]. The overall catalytic mechanism involves two successive methyl transfer reactions from methyltetrahydrofolate to cob(I)alamin and subsequently, from MeCbl to homocysteine (Figure 3). Cob(I)alamin is a reactive intermediate that is sporadically intercepted to generate inactive and oxidized enzyme. Sustained MS activity therefore requires a repair enzyme, methionine synthase reductase (MSR), which is encoded by the *cbfE* locus [26]. Both the *cbfG* and *cbfE* defects lead to a characteristic metabolic profile with presentation of hyperhomocysteinemia, hypomethioninemia, and megaloblastic anemia without methylmalonic aciduria [27].

Since MS can convert cob(II)alamin generated by inadvertent oxidation during turnover to MeCbl in a reaction requiring MSR and *S*-adenosylmethionine (Figure 3), a separate enzyme for the synthesis of MeCbl is not required. Thus, in the model proposed here, cob(II)alamin bound to MMACHC is transferred to MS in a process that is assisted by MMADHC. A histidine residue embedded in the canonical B₁₂-binding D_xH_{xx}G sequence in MS serves as the lower axial ligand to the cobalt, which is bound in the ‘base-off/His-on’ state [28].

MSR, like other dual flavoprotein oxidoreductases viz. cytochrome P450 reductase and nitric oxide synthase can reduce free aquocobalamin to cob(II)alamin [29*,30]. However, only MSR stimulates the formation of holo-MS and this property resides in its FMN-binding domain [30]. A *K_D* of ~4.5 μM has been reported for the interaction between the FMN-binding domain of MSR and the *S*-adenosylmethionine-binding activation domain of MS [31]. An obligatory role for MSR in cofactor docking to MS *in vivo* is not supported by the presence of 84–100% holo-MS in fibroblasts from *cbfE* patients with defects in MSR, which is comparable to the levels seen in control cell lines (49–72%) [32]. It is possible that

complexation between the activation domain of MS and MSR enhances access of the cofactor to the cobalamin-binding domain of MS that is observed under *in vitro* assay conditions [29*,30].

The adenosylcobalamin branch

Tailoring of cobalamin

Inactive cob(II)alamin entering the mitochondrion is tailored into the active AdoCbl form in a reductive adenosylation reaction catalyzed by adenosyltransferase (ATR), encoded by the *cbfB* locus [33,34]. The human enzyme belongs to the PduO family of ATRs whose other members are found in bacteria and are dedicated to AdoCbl synthesis for their target mutases. In the ATR-catalyzed reaction, a reductase furnishes the electron needed for the reduction of bound cob(II)alamin to cob(I)alamin, which then displaces PPPi from ATP to generate AdoCbl (Figure 4). Reduction of cob(II)alamin is thermodynamically very unfavorable but is aided by the 'base-off' conformation which increases the redox potential by ~110 mV [35]. Cob(II)alamin bound to ATR is in the 'base-off' state [36], and in the presence of the cosubstrate, ATP, assumes an unusual four-coordinate 'base-off' conformation [37].

Human ATR is a homotrimer with active sites located at the subunit interfaces. Binding of substrates apparently induces nonequivalence of the active sites and the structure of the human enzyme was obtained with two moles of ATP bound [38]. The better-characterized enzyme from *Methylobacterium extorquens* exhibits weak negative cooperativity and also binds only two moles of AdoCbl per trimer [39**]. Extensive stopped-flow spectroscopic studies combined with kinetic simulations have revealed that ATR is a dual function protein that serves both as an enzyme to tailor AdoCbl and as an escort to deliver the cofactor to the target enzyme, MCM [39**] (Figure 4A). Binding of ATP to the vacant site in cofactor-loaded enzyme leads to the expulsion of one equivalent of AdoCbl [52]. Thus, ATR appears to use a rotary mechanism in which the binding energy of ATP is used to induce a conformational change in an adjacent active site that leads to weakened cofactor affinity and promotes its expulsion. The equilibrium for AdoCbl transfer between the active sites of ATR and MCM is shifted in favor of MCM in the presence of ATP [52].

Delivery of AdoCbl to methylmalonyl-CoA mutase

MCM, encoded by the *mut* locus of cobalamin disorders, catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA in a pathway that funnels catabolites of odd-chain fatty acids, cholesterol, and branched-chain amino acids into the Krebs cycle in mammals [40]. While the human enzyme is a homodimer, many of the bacterial MCMs are heterodimers. MCM exhibits a DXHXXG signature motif for 'base-off/His-on' binding of cobalamin [41,42]. Although MCM and ATR bind AdoCbl in mirror 'base-off' conformations, their coordination environments are distinct, which might be functionally important for mobilizing AdoCbl between the two active sites [43]. Thus, AdoCbl bound to ATR is five-coordinate (or might have a weak sixth ligand such as water) but six-coordinate (with an imidazole ligand) in MCM. This difference in coordination number is reflected in the 40-fold lower K_D for AdoCbl for human MCM versus ATR [43]. In fact, the histidine

residue on MCM that serves as a ligand plays an important role in cofactor transfer from ATR (Figure 4B) and its mutation to alanine or asparagine profoundly impairs AdoCbl transfer [39**]. The role of ATR in cofactor delivery to MCM is supported by the absence of holo-MCM in *M. extorquens* in which ATR has been deleted despite the production of the cofactor in this organism by the *de novo* pathway [44]. The C-terminal region of ATR might be important for its interaction with MCM as its deletion in the *L. reuteri* ATR results in deficient AdoCbl synthesis *in vivo* but not *in vitro* [45].

Editing, gating, and radical protection by MMAA

MMAA (*methylmalonic aciduria type A*) is the locus of mutations associated with the *cbIA* complementation group [46]. Fibroblasts from *cbIA* patients exhibit highly reduced AdoCbl content [47] suggesting an auxiliary role for this protein in AdoCbl genesis. MMAA belongs to the G3E family of GTP-binding proteins, which includes other metallochaperones [48]. A bacterial ortholog, MeaB from *M. extorquens*, has been characterized most extensively and is postulated to play a role in protecting the radical intermediates during MCM turnover [44,49]. Functional [50] and structural [51] studies reveal that MeaB is a homodimer and its N-terminal region is postulated to interact with MCM.

MeaB exhibits a nanomolar affinity for MCM [50] and in the complex, each protein influences the properties of the other. MeaB exhibits low intrinsic GTPase activity which is enhanced ~100-fold in the complex by the GAP (GTPase activating protein) activity of MCM [50]. Similarly, MeaB protects MCM from oxidative interception of its radical intermediates during catalytic turnover and enhances the reaction rate of MCM ~2-fold [49]. We hypothesize that MeaB plays a role in gating the transfer of AdoCbl from ATR to MCM and also functions as an editor, screening inactive (cob(II)alamin) from active (AdoCbl) cofactor during docking thereby averting reconstitution of inactive MCM (Figure 4).

The missing players

The identities of all eight genes giving rise to the *cbIA-G* and *mut* classes of cobalamin disorders are now known and have stimulated biochemical investigations into the intricate pathway for cobalamin processing and targeting. However, these eight gene products do not fully account for the functions that are minimally needed for B₁₂ assimilation into its active cofactor forms. These include a putative lysosomal B₁₂ binder for conveying the cofactor to the LMBD1 transporter, a reductase that partners with MMACHC to decyanate CNCbl, a mitochondrial transporter, and a mitochondrial reductase that assists ATR during the synthesis of AdoCbl. The absence of discrete genetic complementation groups that can be ascribed to these functions might be explained by the rarity of patients with mutations in these genes, or more likely, from the shared use of these proteins with other cellular functions.

Conclusions

Genetic and bioinformatics approaches have fueled rapid gains in recent years in identifying the culpable genes and detecting mutations associated with cobalamin disorders. They have also opened the doors to interrogating the biochemistry of this trafficking pathway in which

minimally, six genes are dedicated to elaborating and shepherding the active cofactor forms to the two mammalian enzymes that utilize B₁₂. The complexity of this pathway underscores the importance of sequestering a rare and reactive cofactor from its point of entry to its place of utilization in target enzymes. Insights into the cellular strategy for escorted delivery of cobalamin might be pertinent to those deployed for trafficking other reactive organic cofactors, viz. pyridoxal phosphate and thiamine pyrophosphate.

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Figure 1.
Structures of cobalamin derivatives and the various ligation states.

Figure 2. Schematic view of the intracellular B₁₂ trafficking pathway. The known and proposed functions of the gene products associated with the complementation groups *cbtA-G* and *mut* are indicated. The question marks highlight some aspects of the trafficking pathway that are poorly characterized.

Figure 3.

Proposed pathway for B₁₂ targeting to methionine synthase for MeCbl synthesis. Cofactor transfer from MMACHC to MS precedes conversion of inactive cob(II)alamin to MeCbl, in a process that requires MSR, NADPH, and AdoMet (*S*-adenosylmethionine). During turnover, B₁₂ bound to MS shuttles between the MeCbl and cob(I)alamin states. Oxidation of the latter creates the dependency of the MS reaction on reductive activation by MSR.

Figure 4.

Mitochondrial components involved in AdoCbl synthesis and targeting. **(A)** ATR converts cob(II)alamin to AdoCbl and transfers the product directly to MCM, in a process that is gated by MMAA (or MeaB in bacteria) and triggered by ATP binding to ATR. MMAA/MeaB is expected to exist in a complex with MCM. **(B)** Schematic representation of how the histidine (shown in stick representation) on a disordered loop in the B₁₂-binding domain of MCM ‘picks’ AdoCbl out of the active site pocket of ATR.