The Import and export of vitamin B12 in bacteria

Nijland, Mark

DOI:
10.33612/diss.975531280

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2024

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 1

Membrane transport of cobalamin

Mark Nijland1#, José Miguel Martinez Felices1#, Chancievan Thangaratnarajah1, Dirk J. Slotboom1*

1 University of Groningen, Faculty of Science and Engineering, Groningen Biomolecular Sciences and Biotechnology, Membrane Enzymology Group, Nijenborgh 4, 9747 AG, Groningen, The Netherlands.

#Contributed equally, *Corresponding author

This chapter was published in *Vitamins and Hormones* 119, 121-148 (2022).
DOI: 10.1016/bs.vh.2022.01.008

Abstract

A wide variety of organisms encode cobalamin-dependent enzymes catalyzing essential metabolic reactions, but the cofactor cobalamin (vitamin B12) is only synthesized by a subset of bacteria and archaea. The biosynthesis of cobalamin is complex and energetically costly, making cobalamin variants and precursors metabolically valuable. Auxotrophs for these molecules have evolved uptake mechanisms to compensate for the lack of a synthesis pathway. Bacterial transport of cobalamin involves the passage over one or two lipidic membranes in Gram-positive and -negative bacteria, respectively. In higher eukaryotes, a complex system of carriers, receptors and transporters facilitates the delivery of the essential molecule to the tissues. Biochemical and genetic approaches have identified different transporter families involved in cobalamin transport. The majority of the characterized cobalamin transporters are active transport systems that belong to the ATP-binding cassette (ABC) superfamily of transporters. In this chapter, we describe the different cobalamin transport systems characterized to date that are present in bacteria and humans, as well as yet-to-be-identified transporters.
1. Introduction

Cobalamin (vitamin B12) is a complex organometallic cofactor belonging to the group of cobamides, and is essential for a large number of organisms from almost all domains of life, with the notable exception of plants. Also fungi were thought not to make use of cobalamin, but a recent publication showed that a few early branching fungi may make use of the cofactor. The structural core of cobamides consists of a corrin ring with a chelated cobalt ion in the center, which is additionally coordinated by a “lower” ligand at the α-axial position and an “upper” ligand at the β-axial position. The lower ligand is usually a covalently attached side chain of the corrin ring, and folds over to coordinate the cobalt ion. Different lower ligands give rise to distinctive cobamides, and include organic moieties that fall into the group of benzimidazoles, purines or phenols. Characteristic for cobalamin is the presence of a 5′6′-dimethylbenzimidazole (DMB) base as lower ligand. While this moiety is generally coordinated to the cobalt ion (base-on conformation) in free cobalamin under physiological conditions, it can dissociate (base-off conformation) depending on temperature, pH, oxidation state of the cobalt ion and protein environment. In the base-off conformation, the cobalt coordination can be taken over by an external ligand, such as a protein side chain. Methylcobalamin and adenosylcobalamin represent the biological active cofactor forms that are used by distinctive cobalamin-dependent enzymes. These cofactors are involved in various metabolic processes, including DNA and RNA synthesis, amino acid metabolism, carbon and nitrogen metabolism and secondary metabolite synthesis. In the process of industrial production of cobalamin, which involves bacterial fermentation, a cyano-group is added as the upper ligand. Cyanocobalamin is commonly used in pharmaceuticals, supplements and food additives, and requires removal of the cyano-group for conversion into the active cofactors methylcobalamin or adenosylcobalamin.

Despite its widespread use as cofactor, de novo biosynthesis of cobalamin is confined to only a limited number of bacterial and archaeal species. This complex synthesis route involves approximately 30 enzymatic steps and can occur by two alternative pathways, differing in oxygen requirements and timing of cobalt insertion (for details, see reviews). Organisms that make use of cobalamin-dependent enzymes, but lack the capacity to synthesize the cofactors themselves, have to acquire cobalamin from external sources (auxotrophs). Humans can only meet their cobalamin requirement by absorbing the complete cofactor from their diet, while various bacteria can also convert precursor molecules (“salvaging”) or alternative cobamides (“remodeling”) taken up from the environment into cobalamin. Often cobalamin producers can also import the cofactor, which is energetically more favorable than the biosynthesis. While knowledge of the biosynthetic pathway of cobalamin and its chemical properties is extensive, insight in how cobalamin or its derivatives or precursors are taken up into the cell is limited. Here, we provide an overview of the characterized membrane transport systems that enable the uptake of cobalamin and its derivatives into the cell. We will be focusing on the systems that are present in bacteria and humans.
Membrane transport of cobalamin

Figure 1 – Structure of cyanocobalamin in the base-on and -off conformation, the variants of the upper ligand to form different chemical forms and lower ligands to form distinct cobamides. Cobalamin is shown in yellow stick representation and illustrates a composite of the base-on (PDB: 1n2z) and -off conformation (PDB: 3bul). Oxygen, nitrogen and phosphate atoms within are colored in red, blue and orange, respectively. The coordinated cobalt ion within the corrin ring is shown as a wine-colored sphere. The upper and lower ligands forming a covalent interaction with the cobalt ion are colored in green and purple, respectively. Chemical structures of variants of the upper (green box) and lower (purple box) ligands are shown with the position forming the covalent interaction with the cobalt ion indicated by a wine-colored sphere and a solid line. Parts of the molecule indicating cobyric acid, cobinamide and cobamide are highlighted.

2. Membrane transport systems in bacteria

Cobalamin is used by bacteria for various metabolic processes and genomic analysis has shown that more than three quarters of the bacteria harbor cobalamin-dependent enzymes. However, more than half of this subset of bacteria lack the genetic capacity to synthesize the cofactor. Moreover, it is not clear whether species that have the genetic capacity, actually produce cobalamin in their natural habitat. For the model organism
Escherichia coli, it is estimated that only approximately 20 cobalamin molecules per cell are already sufficient to support growth in cobalamin-dependent conditions\textsuperscript{15}, which means that high-capacity transport is not needed. Bacteria have evolved various membrane transport systems to mediate the uptake of cobalamin from the environment. The currently identified transporters are described in more detail below.

### 2.1 Transport across the outer membrane in Gram-negative bacteria

A key feature of Gram-negative bacteria is the presence of a double membrane system. In addition to the plasma membrane, Gram-negative bacteria also have an outer membrane, complicating the uptake of molecules into the cell\textsuperscript{16}. Outer membrane proteins play a crucial role in the transport of nutrients across this membrane. Porins and substrate-specific channels are passive transporters that facilitate the diffusion of small molecules (up to 600 Da) into the periplasm\textsuperscript{17}. However, larger molecules or those present in low concentrations in the extracellular environment, such as cobalamin, require active transport systems to cross the outer membrane\textsuperscript{18}. Gram-negative species have evolved TonB-dependent outer membrane transporters (TBDTs) to transport a variety of scarce nutrients, including cobalamin\textsuperscript{18}.

#### 2.1.1 BtuB

BtuB is a member of the TonB-dependent outer membrane transport family, widespread among Gram-negative bacteria, and mediates the active transport of cobalamin and variants into the periplasm (Fig. 2)\textsuperscript{19}. This transporter exhibits a conserved architecture among the TonB-dependent outer membrane transport family, consisting of a 22-stranded β-barrel and an inserted plug domain\textsuperscript{18}.

Transport activity for BtuB has not been demonstrated in vitro using purified protein reconstituted in liposomes, which hampers mechanistic understanding. Although the exact transport mechanism is not well understood, X-ray crystal structures of members of the TonB-dependent outer membrane transport family in different conformational states provide a glimpse of the structural mechanism involved in the translocation of the bound substrate to the periplasm\textsuperscript{18}. The binding site for cobalamin is formed by residues located on the extracellular side of the so-called plug domain in the center of the β-barrel, the extracellular loops and the interior wall of the β-barrel\textsuperscript{20}. Crucially, co-operative binding of calcium ions is essential to support binding of cobalamin to BtuB within nanomolar affinity (Table 1)\textsuperscript{21,22}. The proton motive force across the inner membrane is used as an energy source to facilitate the active transport process, which is transmitted to BtuB via the energy-transducing TonB complex\textsuperscript{18}. This is achieved by the physical interaction of the Ton box, a conserved motif at the periplasmic amino-terminal region of the plug domain, with the TonB complex\textsuperscript{23}. Although it is known that this interaction is essential to release cobalamin into the periplasm, the mechanism of passage through BtuB remains to be elucidated.

Gram-negative bacteria usually utilize a single BtuB transport system to facilitate cobalamin transport\textsuperscript{20}. However, nearly all members of the *Bacteroides* species inhabiting the human gut harbor multiple (up to four) BtuB homologs that are encoded on separate
loci\textsuperscript{24}. Interestingly, they display distinct preferences toward corrinoids that differ in their lower ligand\textsuperscript{24}. Moreover, each homolog contributes differently to the relative bacterial fitness in competitive environments \textit{in vitro} as well as \textit{in vivo}. Whether the presence of multiple corrinoid transport systems simply provides the bacteria with competitive advantages or may play a bigger role in communication between bacterial species remains to be elucidated\textsuperscript{24}.

\textit{Bacteroides} species also encode the accessory protein BtuG within the genetic loci where BtuB is also encoded\textsuperscript{25}. BtuG is a cell-surface exposed lipoprotein that binds cobalamin and cobinamide with femtomolar affinity (187 and 193 fM, respectively), and associates with BtuB to support efficient uptake of the substrate by the accompanying BtuB homolog in \textit{Bacteroides thetaiotaomicron}\textsuperscript{25}. Although BtuB is capable of importing cobalamin on its own, BtuG-mediated cobalamin scavenging is critical for \textit{in vivo} growth of \textit{B. thetaiotaomicron} \textit{in vivo} growth of \textit{B. thetaiotaomicron} \textit{in germ-free mice}\textsuperscript{25}. Remarkably, it was shown that BtuG is able to extract cobalamin from the human carrier protein intrinsic factor (IF), which binds cobalamin with picomolar affinity (1 pM)\textsuperscript{26}, providing \textit{Bacteroides} a competitive advantage for the cobalamin sources present in the human gut (see section on Transport of cobalamin in human)\textsuperscript{25}.

2.2 Transport systems of the plasma membrane in Gram-negative and -positive bacteria

While BtuB is the only identified cobalamin transporter in the outer membrane, the repertoire of membrane transport systems for cobalamin in the plasma membrane of Gram-negative and -positive bacteria is more extensive. Comparative genomic analysis for cobalamin metabolism in bacteria revealed that diverse solutions for cobalamin transport across the plasma membrane exist\textsuperscript{27–29}. All characterized cobalamin transporters in bacteria to date belong to the superfamily of adenosine triphosphate (ATP)-binding cassette (ABC) transporters (Fig. 2), with the exception of BtuM, which is nonetheless structurally related to a part of an ABC transporter. Core to ABC transporters is the presence of two well conserved nucleotide-binding domains or subunits (NBDs) exhibiting ATP binding and hydrolysis activity. The NBDs are connected to transmembrane domains or subunits (TMDs) that contain a substrate translocation pathway\textsuperscript{30}. The energy released upon ATP hydrolysis is used to power conformational changes in the membrane domains, ultimately leading to substrate translocation across the membrane\textsuperscript{31}. Based on high resolution structures obtained by X-ray crystallography and cryogenic electron microscopy (cryo-EM), ABC transporters have been classified into seven different types. While all types make use of related NBDs, the TMDs are structurally diverse and do not show obvious evolutionary relatedness\textsuperscript{30}. The characterized bacterial cobalamin transporters belong to type II, III or IV of ABC transporters, which we will lay out in detail below.

2.2.1 BtuCDF

The cobalamin transporter from \textit{E. coli}, BtuCD-F (Fig. 2), is the best characterized among the cobalamin transporters, and belongs to the type II class of ABC importers\textsuperscript{30}. Homologs are
widely distributed among Gram-negative and -positive bacteria. They are present in 90% of the bacteria that do not encode enzymes for cobalamin synthesis, yet require the vitamin as evidenced by the presence of genes coding for enzymes that use the cofactor cobalamin. Some organisms encode more than one homolog of BtuCD-F within their genome. Moreover, a BtuCD-F homolog has been found in the archaeon *Halobacterium sp. NRC-1*, constituting the first characterized cobalamin transporter in archaea.

BtuCD-F from the Gram-negative bacterium *E. coli* mediates the translocation of cobalamin from the periplasm across the plasma membrane into the cytoplasm. The core is formed by an oligomeric assembly consisting of two identical NBDs (BtuD) and two identical TMDs (BtuC) (Fig. 2). The substrate translocation pathway is found at the interface between the two TMDs BtuC, allowing the passage of cobalamin during the transport process across the membrane. Each copy of BtuD interacts with BtuC on the cytoplasmic side, and hence the NBDs are accessible for ATP from the cytoplasm. A single substrate-binding protein specific for cobalamin, BtuF, interacts with the core complex on the periplasmic side forming the full transporter complex (Fig. 2). Cobalamin binds BtuF in the base-on conformation, and with nanomolar affinity. Such a high binding affinity is an essential property given the scarcity of cobalamin in the extracellular environment. Binding of the cobalamin precursor cobinamide to BtuF and transport by the BtuCD-F complex has also been demonstrated, albeit at lower affinity and transport rates. The wealth of information from crystallographic and functional studies allows a detailed description of the transport cycle of cobalamin.

In absence of cobalamin, the BtuCD-F complex rests in a conformation with the membrane pathway sealed on the cytoplasmic side (“outward-facing” state), from which BtuF partly or completely dissociates revealing its substrate-binding site for cobalamin, thus allowing the capture of cobalamin with high affinity. In presence of ATP, BtuCD retrieves cobalamin from the loaded BtuF, and traps it in a cavity approximately in the center of the membrane by closing off the translocation pathway to both sides of the membrane. ATP hydrolysis triggers conformational changes within the TMDs whereupon the cytoplasmic gate opens and the translocation pathway collapses, resulting in cobalamin being pushed into the cytoplasm. Once released, the cytoplasmic gate closes and the periplasmic gate opens back ready to accept a new cobalamin molecule. A common property among type II ABC transporters is their specificity for low-abundant substrates, as well as a high level of basal or futile ATP hydrolysis. This means that hydrolysis of the majority of ATP molecules is not mechanistically coupled to cobalamin transport. Such futile hydrolysis may be a necessary trade-off for efficient high affinity scavenging of cobalamin from the environment.

### 2.2.2 ECF-CbrT

On the basis of comparative genomic analysis, cobalamin transporters belonging to type III ABC transporters (also named the energy coupling factor (ECF)-type) were identified in a subgroup of bacteria that lack a homolog of BtuCD-F. Notably, this study also identified ECF-CbrT in the archaeon *Methanosphaera stadtmanae*, representing, along with BtuCD-F, the only cobalamin transporters identified in archaea to date. ECF transporters constitute the phylogenetically, structurally and mechanistically distinct type III clade of ABC transporters. Like all ABC transporters, ECF transporters have two NBDs EcfA exhibiting
ATPase activity (Fig. 2), which may be either encoded by the same (EcfA) or two homologous genes (EcfA and EcfA'), or as a fusion protein from a single gene (EcfA-A)\(^43\). The NBDs are associated with a membrane-embedded scaffold protein (EcfT) that is structurally distinct from the TMDs found in BtuCD-F (Fig. 2). Both binding and translocation of the substrate are conferred by a membrane embedded substrate-binding protein termed S-component (Fig. 2), which is structurally unrelated to the TMD EcfT. CbrT binds cobalamin in a site near the extracellular face of the membrane, and then moves the substrate to the cytosol in a unique toppling motion\(^44\). EcfT and the two NBDs form together the ECF or energizing module, which ultimately powers substrate transport by the S-component\(^45\).

ECF transporters can be divided into two subgroups based on their modular nature with respect to their assembly. In group I ECF transporters, the S-component associates exclusively with a dedicated ECF module\(^43\), while in group II ECF transporters the ECF module is shared among multiple S-components that have different substrate specificities\(^43,46\). It is of note that predicted cobalamin-specific ECF transporters can be found in both groups.

In vivo complementation assays with a cobalamin-dependent and BtuCD-F-defective *E. coli* strain, and *in vitro* transport assays with purified and into-liposomes-reconstituted protein, showed that the group II ECF transporter ECF-CbrT from the Gram-positive bacterium *Lactobacillus delbrueckii* is indeed a cobalamin transporter\(^44\). The S-component CbrT binds cobalamin and cobinamide with nanomolar affinity *in vitro* (Table 1) in absence of the ECF module. However, the presence of the energizing module is essential to support transport of the substrates\(^44\), a property also observed for other S-components\(^43,46\).

A crystallographic structure of the full complex of ECF-CbrT was solved, in which the S-component CbrT assumes a toppled conformation (with the alpha-helices lying almost parallel to the membrane) in a substrate-free state\(^44\). A similar conformation has also been found in structures of ECF transporters for other substrates\(^47–50\). Based on this single conformation, it is difficult to derive a model for the transport mechanism. However, the mechanism is likely conserved among related ECF transporters for which more structural states have been resolved. Some of them, such as the folate-specific S-component FolT2, share the same ECF module as CbrT\(^50\).

The structure of the folate-bound S-component FolT1 represents a conformation, in which the substrate binding site is occluded, and located close to the extracellular boundary of the lipid bilayer\(^50\). Both the substrate-bound and -free S-component can dynamically associate with the ECF module, which powers the transport\(^6\). Structural and functional data support the notion that the ECF module provides a favorable environment for the toppling of the S-component by bending and thinning of the membrane\(^51\). This toppling motion brings the bound substrate to the cytoplasmatic side of the cell membrane. Once toppled, the substrate is released form the substrate binding site, which is the conformation that has been observed in X-ray crystal structures of ECF transporters to date\(^44,47–50\). The binding and possibly also hydrolysis of ATP is likely to result in the reorientation of the substrate-free S-component from an inward-facing to the outward-facing state and release from the ECF module for a renewed transport cycle\(^50\).
2.2.3 BtuM

Similar to ECF-CbrT, genomic analysis also helped to reveal the inner membrane protein BtuM as a predicted cobalamin transporter\textsuperscript{29}. Interestingly, BtuM is primarily distributed among bacterial species that do not encode the BtuCD-F homolog, nor any gene encoding the ECF module (Fig. 2)\textsuperscript{7}. While all other previously characterized cobalamin transporters were ATP-powered ABC transporters, \textit{in vivo} complementation assays in \textit{E. coli} revealed that BtuM from \textit{Thiobacillus denitrificans} alone is sufficient to mediate the uptake of cobalamin and cobinamide across the inner membrane\textsuperscript{7}. Notably, the precursor cobinamide has been shown to bind BtuM within nanomolar affinity \textit{in vitro} (Table 1)\textsuperscript{7}. Although experimentally not verified, it is expected that cobalamin also binds with a comparable affinity to BtuM. Moreover, there are also no reports of successful \textit{in vitro} transport assays using the purified protein in liposomes.

Surprisingly, a structure of BtuM determined by X-ray crystallography revealed that the overall fold resembles the S-component of ECF transporters (Fig. 2)\textsuperscript{7}. BtuM has subsequently been classified based on the structural homology as a “solitary” S-component, a subgroup of ECF-type ABC transporters that appears to be able to transport substrates across the membrane without the need for an ECF module and ATP hydrolysis\textsuperscript{43}. Notably, BtuM and CbrT share the same structural framework and overlap in their substrate specificity, but do not show significant similarity on the sequence level\textsuperscript{7}.

Unprecedented among the cobalamin transporters is that the structure of BtuM revealed a bound cobalamin molecule in a base-off conformation (Fig. 2)\textsuperscript{7}. This represents a binding mode that is generally observed for proteins with enzymatic activity\textsuperscript{5,52}, which has not been observed for cobalamin transporters before. In BtuM, the DMB base that serves as \(\alpha\)-axial ligand of the cobalt ion is replaced by a cysteine residue, which is highly unusual for cobalamin binding proteins\textsuperscript{7}. The binding of cyanocobalamin to BtuM is accompanied by the removal of the cyano-group from the \(\beta\)-axial position, which could be considered as enzymatic activity\textsuperscript{7}. The cysteine ligation of the cobalt ion is essential to support the enzymatic removal of the \(\beta\)-ligand. The unexpected enzymatic activity of BtuM has also been confirmed biochemically for the substrate cobinamide\textsuperscript{7}. The biological implications of this enzymatic modification are not understood yet.

Apart from its crystallographic structure, very little is known about the transport mechanism of BtuM. A speculative transport model described a facilitated transport that involves metabolic trapping of the substrate in the cytoplasm\textsuperscript{7}. In this model BtuM would bind its substrate from the periplasm and topple over in the membrane. It is unclear how the substrate would be released into the cytoplasm, but since no NBDs are involved to power this process using ATP, it is possible that the release is very slow. To prevent rebinding to the transporter, the released cobalamin may be trapped by binding to enzymes, shifting the equilibrium toward the uptake. With this transport system it is expected that chemical modification of the substrate leads to lower transport rates compared to full ABC transporters, but survival of the cell would still be possible due to the minimal requirements of the vitamin for biological processes\textsuperscript{7}.
2.2.4 Rv1819c

Random in vivo mutagenesis screening in the pathogenic bacterium Mycobacterium tuberculosis revealed that the disruption of the gene for Rv1819c, encoding an ABC transporter, leads to abolished cobalamin uptake\textsuperscript{27}. Rv1819c belongs to the Type IV ABC transporters that includes many members with exporter functions (e.g., multidrug exporters), but recently was also found to contain members with importer functions\textsuperscript{30}. Rv1819c is a homo-dimeric membrane complex, where each protomer consists of a TMD that is covalently linked to a NBD (Fig. 2)\textsuperscript{53}. The main difference between Rv1819c and the other bacterial cobalamin-specific uptake systems BtuCD-F, ECF-CbrT and BtuM is that Rv1819c operates as a multi-solute transporter that facilitates the uptake of multiple unrelated hydrophilic substrates including cobalamin\textsuperscript{53,54}.

The poly-specific nature of the transport system arises from two important structural characteristics. First, in contrast to type I–III ABC importers\textsuperscript{30}, Rv1819c does not make use of a substrate binding protein to confer substrate specificity on the transport system and to bind the substrate with high affinity\textsuperscript{53}. Second, in the structure that was solved, an enormous occluded cavity with a volume over 7700 Å\textsuperscript{3} was found that spans the entire thickness of the membrane\textsuperscript{53}. The surface of this cavity consists of mainly polar and negatively charged residues that favors the interaction of hydrophilic molecules, which may explain its poly-specificity\textsuperscript{53}. Noteworthy is that the cavity is the largest found among the characterized ABC transporter systems and is nearly seven times larger than the internal cavity found in the cobalamin transporter system BtuCD-F, which can only accommodate one cobalamin molecule\textsuperscript{39}. It was speculated that the cavity opens in an alternating fashion to the outside and the cytosol, such that cobalamin may diffuse across the membrane\textsuperscript{53}. However, more work is needed to substantiate this model.

So far, it is evident that Rv1819c not only mediates cobalamin uptake, but also supports the uptake of the antimicrobial peptides bleomycin and Bac7, which are structurally unrelated substrates\textsuperscript{53,55}. Consistent with the poly-specificity of the transporter, Rv1819c does not seem to have a high affinity binding site for cobalamin, which is an important aspect of the above-mentioned dedicated cobalamin transporters to support efficient uptake of the scarce vitamin\textsuperscript{53}. Although Rv1819c is expected to transport cobalamin less efficiently than the dedicated uptake systems, physiologically it may allow uptake of cobalamin at a sufficient level for \textit{M. tuberculosis}, which is an extremely slow-growing bacterium that only requires minute amounts of cobalamin.

2.2.5 BtuN

BtuN was identified by conserved positional linkage of the encoding gene to the \textit{btuB} gene and has been implicated to be involved as a potential candidate in BtuCD-independent transport of cobalamin\textsuperscript{29}. While BtuN was predicted to contain four transmembrane helices\textsuperscript{29}, information on its structure and function are not known to date, and transport activity has not been confirmed.
Figure 2 – Structures of bacterial and mammalian cobalamin transporters. Models of bacterial (green box) and mammalian (purple box) cobalamin transporters are shown in cartoon representation, and are colored according to common structural features: green for substrate translocation pathway, red or orange for nucleotide-binding domains/proteins, yellow for substrate binding proteins, blue for the scaffold protein in ECF transporters, purple for structural features of unknown function. Lighter shades indicate the different protomers within the homo-dimeric assembly. Bound cobalamin molecules are shown in stick representation and the base-on and -off conformations are indicated with labels. The lipid membrane is indicated as a gray bar and the type and orientation of the lipid membrane is indicated. PDB codes of the individual models are indicated under the protein labels.

2.3 Cobamides in microbial communities: Are exporters needed?

Bacteria are usually part of microbial communities, and their fitness is shaped by the flow of nutrients among each other within dynamic physical and chemical networks to fuel metabolism. Cobamides are considered as precious commodities placed at the core of microbial interactions, and comparative genomics analyses estimate that about 85% of...
Membrane transport of cobalamin

bacteria utilize cobamides as coenzyme\textsuperscript{13}. Notably, cobamides that differ in the lower ligand structure are not functionally interchangeable in bacteria and therefore the distribution of specific bacterial species within microbial communities and environments is modulated by the presence of diverse sets of cobamides that these bacteria utilize, and constrained by the presence of a subset of bacteria capable of cobamide \textit{de novo} synthesis. These interactions are either shaped by competition of a micronutrient limited in availability, by utilizing metabolic partnerships between microbes to gain access to a micronutrient that none of the individual microbe is capable to metabolize on its own (syntropy), or by making intermediary or end products available to other microbes within a community (cross-feeding)\textsuperscript{4}. However, all types of interactions within a microbial community requires transport systems that enable the exchange of cobamides between its producers and consumers. While different systems for the import of exogenous cobamides have been identified and characterized in Gram-negative and -positive bacteria (see above), it is not known how synthesized cobamides are released into the surrounding environment. Membrane transport systems for the active export of cobalamin in bacteria have not been identified to date. The occasional lysis of bacteria mediated by the action of bacteriophages or bacteriocins and also by sporulation may therefore serve as mechanisms during which cobamides are made available to others within a microbial community\textsuperscript{56}.

3. Transport of cobalamin in humans

The capacity to produce cobalamin has not made the transition to eukaryotes. It is an essential cofactor for only two enzymes in humans: the cobalamin-dependent methionine synthase that catalyzes the methylation of homocysteine in the cytoplasm, and the mitochondrial methylmalonyl-CoA mutase that catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA\textsuperscript{57,58}. Humans obtain cobalamin exclusively from their diet, where animal products such as meat, fish and dairy products are the primary natural source of the essential vitamin\textsuperscript{59}. The absorption and subsequent distribution of cobalamin to the sites of utilization in the human body is more complex than the uptake systems described for bacteria. The uptake of cobalamin in humans involves multiple carrier proteins, receptors and membrane transport proteins (Fig. 3)\textsuperscript{60,61}. The dysfunction of any of these proteins may result in cobalamin deficiency\textsuperscript{62}. Here, we will focus on what is currently known about the membrane transport systems that mediate the translocation of cobalamin across membranes inside the cell. To provide some context of these transport systems, we briefly describe the absorption and distribution process for cobalamin in humans, but for details refer to extensive reviews\textsuperscript{60–62}.

In humans, three homologous carrier proteins, haptocorrin (HC), gastric intrinsic factor (IF) and transcobalamin (TC), play essential roles in escorting cobalamin through the extracellular fluids. HC and IF protect the vitamin from enzymatic degradation and non-catalyzed breakdown due to extreme pH values\textsuperscript{63}. In the stomach, HC binds cobalamin after its release from food and aids in the passage toward the duodenum. HC is subsequently degraded by pancreatic proteases in the duodenum, where the difference in pH favors binding of cobalamin to the carrier protein IF\textsuperscript{64}. The cobalamin-IF complex is then recognized by the cubam receptor in the distal ileum, which facilitates the internalization into the enterocytes through receptor-mediated endocytosis\textsuperscript{65}. IF is degraded in the lysosome of
enterocytes, which liberates cobalamin in the lysosomal lumen. The actual membrane transport step from the lumen to the cytosol across the lysosomal membrane is mediated by the ABC transporter ABCD4. The fate of cobalamin is threefold: either it serves as a cofactor in the cobalamin-dependent methionine synthase in the cytoplasm, or it is imported into mitochondria via an unknown transporter to serve as a cofactor in methylmalonyl-CoA mutase, or it is transported from the basolateral side of the cell into the bloodstream by the ABC transporter MRP1. In blood plasma, cobalamin is bound to either the carrier protein HC or TC, where the former is responsible for excretion of inactive versions of cobalamin, and the latter for cellular delivery to the cells throughout the body. The cobalamin-TC complex is recognized by the ubiquitous receptor CD320 and subsequently internalized by endocytosis, after which TC is degraded to eventually end up again with free cobalamin in the lysosome. Only in the kidney, the internalization of the cobalamin-TC complex is mediated by megalin, an alternative receptor to CD320.

Figure 3 – Uptake of cobalamin by enterocytes from the distal ileum and posterior utilization and systemic transport. Cobalamin-bound IF is recognized by the CUBAM receptor of the enterocyte in the distal ileum. The endocytosed complexed IF is subsequently proteolyzed and free cobalamin imported to the cytoplasm by the transporter ABCD4. Cytoplasmic cobalamin can then be utilized in the enterocyte metabolism as a cofactor for MTR (in the methyl-cobalamin variant), transported to the mitochondrion via a yet-to-be-discovered mitochondrial transporter (where it is utilized by MCM as adenosyl-cobalamin) or exported towards the plasma by MRP1 or another unknown transporter. In the bloodstream, free cobalamin is predominantly captured by TC, which delivers the vitamin throughout the system. Cobalamin-bound TC is recognized by the CD320 receptor. Once imported, cytoplasmic cobalamin undergoes the same fate as in the enterocyte. Abbreviations: MTR: methionine synthase, MCM: methylmalonyl-CoA.

3.1 Lysosomal cobalamin transporter ABCD4

Genetic analysis of cobalamin deficient patients revealed that mutations in the genes encoding the ABC transporter ABCD4 and the integral membrane protein LMBD1 are associated with a defective release of cobalamin from the lysosome. The mutations in the genes are also known as the cause of the cobalamin metabolism disorders cblJ and cblF, respectively.
ABCD4 belongs to the type IV ABC transporters and is a homo-dimer, where each protomer contains one TMD with six transmembrane helices followed by a cytosolic NBD (Fig. 2). The protein imports the internalized cobalamin from the lysosomal lumen into the cytoplasm (Fig. 3). While purified ABCD4 alone was shown to mediate cobalamin transport across in liposomes (Table 1), LMBD1 is essential to facilitate the transport process across the lysosomal membrane in vivo, where it plays an important role in lysosomal targeting of ABCD4. Defects in LMBD1 or ABCD4 that preclude interaction between the two proteins or mislocalization of LMBD1 affect transport of cobalamin across the lysosomal membrane. Both proteins have been shown to interact in vivo and in vitro with sub-nanomolar dissociation constants. The structure of ABCD4 was resolved by cryo-EM (Fig. 2), revealing a fold similar to the distant homolog Rv1819c of M. tuberculosis (Fig. 2). The transporter was captured in an open conformation with the binding site exposed to the lysosomal lumen. Although more structures would be needed to elucidate the transport mechanism, the characterization of similar type IV ABC transporters suggest an alternating access transport mechanism. The structure of ABCD4 has not revealed a specific binding site for cobalamin, which opens the possibility that there are similarities in the transport mechanism with Rv1819c, but more work is needed to substantiate possible mechanistic similarity.

3.2 MRP1

Upon the transport of cobalamin from the lysosomal lumen into the cytoplasm, cobalamin is utilized within the cell or released from the basolateral side of the enterocyte into the bloodstream (Fig. 3). No cobalamin-specific human transporter has been reported to perform the latter function. Instead, the multidrug resistant protein MRP1, which functions as a non-specific ABC exporter, has been shown to mediate the export of cobalamin into the medium of human and murine cultured cells. MRP or MRP-like proteins are present in all eukaryotic organisms, but are absent from bacteria and archaea.

MRP1 is a versatile transporter, which belongs to the type IV ABC transporters. However, unlike the structurally related Rv1819c and ABCD4, which are importers, MRP1 functions as an exporter. MRP1 is encoded by a single peptide consisting of two NBDs, two TMDs forming the transport path, and an additional, membrane embedded N-terminal domain of unknown function consisting of five transmembrane helices (Fig. 2).

The substrates recognized by MRP1 are diverse. However, they share a common amphiphatic organo-acidic nature, typically containing large hydrophobic groups. In humans, MRP1 has a dual “good-evil” role regarding its function as a multidrug exporter. While its widespread presence in the tissue-blood interface provides protection against toxic molecules, its expression also impedes chemotherapy and the treatment of a variety of brain disorders.

Structural and functional analysis of MRP1 revealed a substrate-driven transport mechanism, where the transporter rests in an inward-open state, with the NBDs separated from each other, which impedes ATP hydrolysis and provides access to the substrate-binding cavity between the two TMDs. Substrate interaction brings the TMDs together, which also leads to closer proximity of the NBDs, enabling ATP hydrolysis to export the bound substrate toward the extracellular space.
In vivo, knockdown mice for the MRP1 homolog showed decreased cobalamin levels in blood and tissue\textsuperscript{80}. However, these mice did not present the characteristic metabolic markers for cobalamin deficiency\textsuperscript{80}. Consistent with these findings, sequence analysis of cobalamin deficient patients that could not be explained by malabsorption of cobalamin did not present severe mutations in the gene encoding for MRP1 either\textsuperscript{86}. As suggested by Shah et al., these findings point toward at least another exporter implicated in cobalamin efflux from mammalian cells (Fig. 3)\textsuperscript{86}, preventing MRP1-defective individuals from developing cobalamin-related diseases and inducing cobalamin malabsorption-like phenotype even when MRP1 is fully operational\textsuperscript{80}. Further investigations are needed to clarify the exact role of this potential transporter.

### 3.3 Mitochondrial cobalamin transporter

It is apparent that cobalamin has to enter the mitochondrion as methylmalonyl-CoA mutase activity is dependent on cobalamin as cofactor\textsuperscript{87}. However, it is not clear how cobalamin is imported into mitochondria. While the passage of cobalamin across the mitochondrial outer membrane is likely to be mediated by the voltage-dependent anion channel (VDAC), which enables the exchange of small molecular-mass solutes up to 5 kDa between the cytoplasm and the mitochondrial intermembrane space\textsuperscript{88}, it is unclear how the molecule passes the inner membrane. As the mitochondrial inner membrane is impermeable to ions and molecules, the passage of cobalamin must be mediated by a transporter. Nonetheless, the identity of the transporter that facilitates the import of cobalamin is unknown. A candidate for cobalamin import into mitochondria has been described in knockout studies of multiple ABC transporters in the roundworm *Caenorhabditis elegans*\textsuperscript{89}. The *wht-6* gene product encoding an ABC transporter has been suggested, but the disruption of the *wht-6* gene did not result in a complete loss of transport and direct evidence of mitochondrial localization and cobalamin transport is currently lacking\textsuperscript{89}. As many of the transport steps of structurally diverse sets of molecules across the mitochondrial inner membrane are facilitated by members of the SLC25 family of mitochondrial carriers\textsuperscript{90}, the import of cobalamin could potentially be mediated by one of the unidentified transporters within this family.

### 4. Concluding remarks

Cobalamin plays an essential role in metabolism as a cofactor in organisms from all domains of life. Especially in the last few years, great progress has been made in the structural and biochemical characterization of cobalamin transporters. These have remarkably advanced our understanding of cobalamin transport and highlight that the proteins share common features, yet have evolved different solutions that facilitate the uptake and transport of cobalamin and its derivatives across lipid membranes. More work is however required to fully understand and describe the transport mechanism of many cobalamin transporters. While almost all cobalamin transporters characterized to date are powered by ATP, other mechanisms of transport may be uncovered among the yet-to-be-identified cobalamin transporters in the future.
Table 1 – Available functional data of cobalamin transporters

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Organism</th>
<th>Cobalamin substrates</th>
<th>Kinetics</th>
<th>Futile ATP hydrolysis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No NBDs (TonB-dependent)</td>
<td>(1) (Bradbeer et al., 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2) (Mills et al., 2016)</td>
</tr>
<tr>
<td><strong>Bacterial outer membrane transporters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BtuB</td>
<td><em>Escherichia coli</em> (Gram-negative)</td>
<td>CN-Cbl: Kd ~ 0.3 nM (on bacterial membranes) (1), depletion of Ca2+ reduces affinity by 50 to 100-fold (1). CN-Cbl: Kd ~ 0.8 nM (competitive transport assay against labelled CN-Cbl (1))</td>
<td>CN-Cbl: kcat = 8.6 × 10^-3 ± 0.7 min^-1 (2) kcat = 1.5 × 10^-3 ± 0.5 min^-1 (in presence of TonB (1))</td>
<td>ATPase rate = 180 nmol min^-1 mg^-1 (BtuCD) (2)</td>
<td>(1) (Rempel et al., 2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bacterial cytoplasmic membrane transporters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BtuCDF</td>
<td><em>Escherichia coli</em> (Gram-negative)</td>
<td>CN-Cbl: Kd ~ 9.1-15 nM (BtuF in solution) (1, 2) CN-H2O-Cbl: Kd = 40 ± 10 nM (BtuF in solution) (3)</td>
<td>CN-Cbl: Vmax ~ 0.3 nmol mg^-1 min^-1 (2)</td>
<td>ATPase rate = 440 nmol min^-1 mg^-1 (BtuF) (2)</td>
<td>(1) (Cadieux et al., 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2) (Borths et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3) (Mireku et al., 2017)</td>
</tr>
<tr>
<td>CbtT</td>
<td><em>Lactobacillus delbrueckii</em> (Gram-positive)</td>
<td>CN-Cbl: Kd = 9.2 ± 4.5 nM (ITC on bacterial membranes) (1) OH-Cbl: Kd = 9.6 ± 6.9 nM (ITC on bacterial membranes) (1) Met-Cbl: Kd = 4.5 ± 0.3 nM (ITC on bacterial membranes) (1) diCN-Cbl: Kd = 36 ± 15 nM (ITC on bacterial membranes) (1)</td>
<td>CN-Cbl: Kd = 2.1 ± 0.4 nM (1) Vmax = 0.06 ± 0.01 nmol mg^-1 s^-1 (1)</td>
<td>Not directly tested (futile ATPase activity on the related folate ECF transporter FolT2) (2)</td>
<td>(1) (Santos et al., 2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2) (Swier et al., 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BtuM</td>
<td><em>Thiobacillus denitrificans</em> (Gram-negative)</td>
<td>CN-Cbl: (growth assay) (1) OH-Cbl: (growth assay) (1) diCN-Cbl: Kd = 0.58 - 0.65 µM (ITC on solubilized BtuM) (1) Kd = 5.6 ± 2.8 µM (ITC on solubilized BtuM805 mutant) (1)</td>
<td>diCN-Cbl: t1/2 = 12 ± 0.7 minutes (decyanation) (1)</td>
<td>No NBDs</td>
<td>(1) (Rempel et al., 2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rv1819c</td>
<td><em>Mycobacterium tuberculosis</em> (Gram-negative)</td>
<td>Cobalamin (growth assay, CN-Cbl) (1)</td>
<td>Not determined</td>
<td>Yes (1)</td>
<td>(1) (Rempel et al., 2020)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Human membrane transporters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCD4</td>
<td><em>Homo sapiens</em> (variant not specified) (1, 2)</td>
<td>Cobalamin (growth assay, CN-Cbl) (1)</td>
<td>Kd = 426 µM (1) Vmax = ~ 667 pmol min^-1 mg^-1 (1)</td>
<td>ATPase rate ~ 4.5-11.8 nmol min^-1 mg^-1 (1, 2)</td>
<td>(1) (Kitai et al., 2021)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2) (Xu et al., 2019)</td>
</tr>
<tr>
<td>MRP1</td>
<td><em>Homo sapiens</em> (variant not specified) (2)</td>
<td>OH-Cbl: Kd = 23 µM (1) GS-Cbl: Kd = 6.2 ± 1.6 µM (competitive inhibition against E217βG) (1)</td>
<td>OH-Cbl: Kd = 23 µM (1) Vmax = 76 pmol mg^-1 min^-1 (1)</td>
<td>ATPase rate ~ 8 nmol mg^-1 min^-1 (+1 mM cobalamin) (1)</td>
<td>(1) (Beedholm-Ebsen et al., 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2) (Mao et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3) (Johnson &amp; Chen, 2017)</td>
</tr>
</tbody>
</table>

Abbreviations: CN-Cbl: cyanocobalamin, OH-Cbl: hydroxocobalamin, Met-Cbl: methylcobalamin, diCN-Cbl: dicyanocobamide, CN-H2O-Cbl: cyano-aquocobamnide. Experiments in artificial membranes were preferentially selected, unless otherwise stated.
References


Chapter 1


