Chapter 2

Functional and structural characterization of an ECF-type ABC transporter for vitamin B12

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Abstract

Vitamin B12 (cobalamin) is the most complex of the B-type vitamins and is synthetized via an intricate pathway exclusive to a limited number of prokaryotes. Its biological active variants methyl- and adenosyl-cobalamin contain rare organometallic bonds, which are exploited by enzymes in a variety of central metabolic pathways such as L-methionine synthesis and ribonucleotide reduction. Although its biosynthesis and role as co-factor are well understood, knowledge about uptake of cobalamin by prokaryotic Vitamin B12 auxotrophs is scarce. Here, we characterize a cobalamin-specific ECF-type ABC transporter from Lactobacillus delbrueckii, ECF-CbrT, and demonstrate that it mediates the specific, ATP-dependent uptake of cobalamin. We solved the crystal structure of the ECF-CbrT complex in an apo inward-facing conformation to 3.4 Å resolution. Comparison with the ECF transporter for folate (ECF-FolT2) from the same organism reveals how the identical ECF module adjusts to interact with the different substrate binding proteins FolT2 and CbrT. Although ECF-CbrT is unrelated to the well-characterized vitamin B12 transporter BtuCDF, their biochemical features indicate functional convergence.
Introduction

Vitamin B12 or cobalamin (Cbl) is regarded as the largest and most complex biological ‘small molecule’. The molecule consists of a corrin ring chelating a cobalt ion using four equatorial coordinating nitrogen atoms (Figure 1 and Suppl. Figure 1). At the $\alpha$-axial position the cobalt ion is coordinated by the 5,6-dimethylbenzimidazole (DMBI) base that is linked covalently to the corrin ring. Located at the $\beta$-axial position is the sixth coordinating moiety (1, 2). This ligand can vary among cobalamin derivatives and forms a rare organometallic covalent bond, which offers unique catalytic properties to enzymes that use Cbl as co-factor. The two most common biological active variants have a methyl- or 5’-deoxyadenosyl group resulting in methyl- and adenosyl-cobalamin (Met-Cbl and Ado-Cbl, respectively) at this position. In the industrially produced variant a cyano-group (CN-Cbl) binds at the $\beta$-axial position, and a hydroxy group (OH-Cbl) is present in the degradation product (1).

Enzymes that use Cbl as their co-factor catalyze mostly methyl group transfer reactions, or a variety of different radical-mediated reactions (1, 3). The most prominent example for methyl group transfer is MetE, the Cbl-dependent L-methionine synthase, which uses Met-Cbl to transfer a methyl group onto L-homocysteine to produce L-methionine. The methyl group on Cbl is subsequently restored from methyl-folate (1, 4).

The ability to synthesize Cbl *de novo* is restricted to prokaryotic species in only ~20 genera. Two routes for *de novo* synthesis have been established (aerobic or anaerobic), each requiring approximately 30 different enzymes, which makes *de novo* synthesis a very energy consuming process, and could explain why roughly two thirds of prokaryotes that require Cbl cannot synthesize the molecule and hence depend on uptake (1, 2). For some microbial communities, for instance in the Ross Sea, it has been shown that Cbl production is the limiting factor for biomass production, which generates a demand for Cbl uptake systems among Cbl auxotrophs (5).

In contrast to the well-characterized chemical properties of Cbl, as well as its biosynthesis and role in many enzymatic reactions, the uptake of the vitamin by bacteria is poorly understood. The only characterized Cbl uptake system is the *Escherichia coli* BtuCDF ATP binding cassette (ABC) transporter, which was first described in 1980 (6). Substantial
understanding of the system has been obtained through a combination of biochemical and structural studies (7–11). The importer uses the periplasmic substrate-binding protein BtuF to capture Cbl or its precursor cobinamide (Cbi) with high affinity ($K_D$ values of $\sim 10$ nM and $\sim 40$ nM, respectively) (12, 13). Transport is powered by hydrolysis of ATP by the two BtuD subunits located on the cytoplasmic side of the membrane (14). The substrate passes through the membrane at the interface between two copies of the transmembrane protein, BtuC (7, 9). BtuCDF homologs are found widely in prokaryotes, but they are absent from a subset of bacteria that require uptake of the vitamin (15).

An in silico study by Rodionov et al. in 2009 (15) predicted that the energy coupling factor (ECF-) type ABC transporter ECF-CbrT might be a Cbl transporter (15). ECF-transporters are multi-subunit membrane complexes that consist of two ATPases, similar to the ATPases of ABC transporters, and two membrane embedded proteins, not related to any other protein.
family (16). The two ATPases and one of the transmembrane proteins, EcFT, form the ‘energizing unit’ or ‘ECF-module’. The other membrane protein termed S-component acts as the substrate binding protein and dynamically associates with the ECF-module to allow for substrate translocation. In so-called group II ECF transporters multiple S-components specific for different substrates interact with the same ECF module (17–21). For instance, in Lactobacillus delbrueckii eight different S-components are predicted to share a single ECF module, one of which, CbrT, was predicted to be specific for Cbl (15, 22).

In this work, we biochemically and structurally characterize the ECF-CbrT complex from L. delbrueckii. We demonstrate that ECF-CbrT is a Cbl transporter that catalyzes ATP-dependent uptake of Cbl and its
Functional and structural characterization of an ECF-type ABC transporter for vitamin B12 precursor Cbi. We show that the S-component CbrT mediates high affinity substrate-specificity for Cbl and Cbi, and we report the crystal structure of ECF-CbrT from L. delbrueckii at 3.4 Å resolution in its apo inward-facing state. Although ECF-CbrT is structurally and mechanistically unrelated to BtuCDF, the kinetic parameters of the two transporters are very similar, suggestive of functional convergence.

Results

Expression of ECF-CbrT complements an Escherichia coli strain lacking its endogenous vitamin B12 transporter

To demonstrate that ECF-CbrT is a vitamin B12 transporter we constructed an Escherichia coli knock-out strain with three genomic deletions: ΔbtuF, ΔmetE, and ΔbtuC::KmR (E. coli ΔFEC) (23–25). A similar strain was previously used by Cadieux et al. (13) to identify the substrate binding protein BtuF. The knock-out strain lacks the L-methionine synthase MetE (26). E. coli possesses two L-methionine synthases, MetE and MetH. MetH uses Cbl as cofactor, whereas MetE is not dependent on the vitamin (26, 27). Thus, deletion of metE makes E. coli dependent on Cbl for the synthesis of L-methionine. Because btuF and btuC are also deleted in E. coli ΔFEC, endogenous Cbl-uptake mediated by BtuCDF is abolished (13), and (heterologous) expression of an active Cbl transporter is required to synthesize methionine.

We studied the growth of the deletion strain transformed with an expression plasmid for either BtuCDF (positive control), or an empty plasmid (negative control), or CbrT with or without the ECF module. We grew cells in 96-well plates using minimal medium supplemented with L-methionine or Cbl and monitored the optical density at 600 nm (OD600). In the presence of L-methionine, all strains grew readily, with a lag phase of 300 – 450 minutes (Figure 2a-d) (28). The strains expressing BtuCDF or ECF-CbrT showed similar growth characteristics (lag time of 380 minutes and 470 minutes, respectively) in medium containing Cbl instead of methionine (Figure 2a and 2c, respectively), whereas the deletion strain expressing isolated CbrT (without its cognate ECF-module) did not show substantial growth in the absence of L-methionine when supplemented with Cbl (Figure 2d). The results demonstrate that the full
ECF-CbrT complex constitutes a new Cbl transporter. ECF-CbrT also supported growth of *E. coli* ΔFEC in the presence of Cbi instead of Cbl, albeit with a longer lag-time (730 minutes, Figure 2c), indicating that Cbi is a transported substrate of ECF-CbrT. We hypothesize that the longer lag-time is due to the extra time required to express the necessary enzymes for Cbl synthesis from Cbi (29). Our further *in vitro* work shows that ECF-CbrT indeed supports efficient Cbi transport (see below).

**ECF-CbrT catalyzes ATP-dependent transport of cobalamin and cobinamide**

We purified ECF-CbrT, reconstituted the complex in liposomes and assayed for the uptake of radiolabeled Cbl (*^57^Co-cyanocobalamin*). Uptake of radiolabeled Cbl into the proteoliposomes was observed only when the proteoliposomes were loaded with Mg-ATP and not when Mg-ADP was incorporated (Figure 3a). While this experiment shows that transport is strictly ATP-dependent, similar to what was found for other ECF transporters (20, 22), the ratio between ATP molecules hydrolyzed and Cbl molecules transported cannot be derived from this data. To obtain this ratio, simultaneous measurements of Cbl uptake and ATP hydrolysis rates...
are needed, which is technically difficult. Additionally, the related ECF transporter for folate displays are large extent of futile ATP hydrolysis (not coupled to transport (22), which further complicates the determination of the coupling ratio. Nonetheless, the Ecfa and Ecfa’ subunits contain all the motifs to form functional ATPases, and therefore we speculate that transport of Cbl is coupled to the hydrolysis of two ATP molecules. Using an ATP concentration of 5 mM, the apparent $K_M$ for Cbl uptake was $2.1 \pm 0.4$ nM and the $v_{max} = 0.06 \pm 0.01$ nmol mg$^{-1}$ s$^{-1}$ (Figure 3 and Suppl. Figure 2). To test the substrate specificity of the new vitamin B12 transporter, we conducted uptake experiments with a variety of competing compounds that are structurally similar to Cbl (Figure 3b). Addition of a 250-fold excess of unlabeled CN-Cbl inhibited the uptake of the radiolabeled substrate almost completely. Met-Cbl and OH-Cbl inhibited uptake to a similar extent as CN-Cbl, whereas Ado-Cbl was less effective (inhibition to $\sim$25%). Addition of a 250-fold excess of Cbi also decreased the uptake of radiolabeled Cbl to $\sim$25% (Figure 3b). To test whether Cbi is a transported substrate (that competitively inhibits transport of Cbl) or a non-transported compound that can only bind to the transporter, we directly measured uptake of Cbi (Figure 3a). Radiolabeled Cbi is not commercially available and, therefore, we synthesized the compound by treating $^{57}$Co-cyanocobalamin with perchloric acid (30). The complete conversion of Cbl into Cbi was confirmed by mass spectrometry. Cbi was transported into liposomes containing ECF-CbrT, and transport required luminal Mg-ATP, confirming that Cbi is a transported substrate (Figure 3a). Finally, we tested whether hemin inhibits Cbl transport (Figure 3b). Hemin and Cbl are structurally related and share the same precursor uroporphyrinogen-III (31), but unlike Cbl, hemin consists of a flat porphyrin ring with a chelated iron ion, and has a chloride ion as one of the axial ligands. Hemin did not compete with Cbl-uptake (Figure 3b), showing that, although promiscuous among Cbl variants and Cbl-precursors, ECF-CbrT is a dedicated vitamin B12 transporter.

We aimed to obtain further biochemical information on the isolated S-component CbrT. We could purify CbrT only in the substrate-bound state (Figure 4a) The protein without substrate was unstable in detergent solution and prone to aggregation. Apparently, substrate binding had a stabilizing effect on CbrT, an observation that has been made more often for membrane proteins (20, 21).
From the spectral properties of Cbl that was co-purified with CbrT, we conclude that CbrT binds Cbl in a ~1:1 ratio in detergent solution (Figure 4a), which reflects the common substrate to protein stoichiometry for S-
components (21, 22, 32). Because we could not obtain purified apo CbrT, we studied the substrate-binding affinity using *E. coli* crude membrane vesicles (CMVs) containing overexpressed apo CbrT. Isothermal titration calorimetry (ITC) revealed binding of both CN-Cbl and Cbi with dissociation constants of 9.2 ± 4.5 nM and 36 ± 15 nM, respectively (*Figure 4b, c and e*). As a negative control, CMVs without CbrT were used to exclude unspecific binding of CN-Cbl or Cbi (*Figure 4d*). Cbl analogues, OH-Cbl and Met-Cbl, were also probed with ITC and found to bind to CbrT with similar binding affinities like CN-Cbl (*K_D* values of 9.6 ± 6.9 nM or 4.5 ± 0.3 nM respectively, *Figure 4 and Suppl. Figure 3*), supporting the notion that CbrT is promiscuous towards the β-axial ligand of Cbl and corroborating the findings of the competition assay.

Although the use of CMVs precluded the determination of the number of binding sites (the concentration of CbrT in the CMVs is unknown), the thermodynamic values (*K_D*, Δ*H*, and Δ*S*) derived from the ITC measurements do not depend on this number. Assuming that CbrT has a single substrate binding site (consistent with the spectral properties, *Figure 4a*), the expression level of CbrT in the membranes could be calculated and we found that CbrT accounted for ~0.9% (w/w) of the protein content in the membrane.

*Structure of the vitamin B12-specific ECF transporter in its apo and post substrate-release state*

We crystallized the ECF-CbrT complex in detergent (n-Dodecyl-β-D-maltopyranoside, DDM) solution and solved a crystal structure to 3.4 Å (*Figure 5a*) using molecular replacement with the structure of the folate transporter, ECF-FolT2, from *L. delbrueckii* as a search model (22). Statistics of data collection and structure refinement are summarized in Suppl. Table 1. There are two copies of ECF-CbrT in the asymmetric unit, corresponding to molecules A and B, each of them comprising CbrT, EcfT, EcfA, and EcfA’.

The identical ECF modules of the ECF-FolT2 and ECF-CbrT complexes have very similar overall structures, with a few notable conformational differences (*Figure 5b*). In both complexes, the two nucleotide-binding domains (NBDs, called EcfA and EcfA’) are in a nucleotide-free state, adopting an open conformation with two incomplete ATP-binding sites. With the NBDs of ECF-FolT and ECF-CbrT aligned structurally (rmsd
1.8Å), the coupling helices of EcfT, which transmit the conformational changes in the NBDs upon ATP-hydrolysis to the membrane domains, also superimpose well between the two complexes (rmsd 1.5Å). However,
the transmembrane-helices of EcfT adopt different conformations (rmsd 4.4Å). They are offset like rigid bodies, hinging approximately around Pro71 (Figure 5b). Structural flexibility of the membrane domain has been observed before (22, 33) and is likely necessary to accommodate different S-components, and may facilitate toppling of the S-components during the catalytic cycle (22).

The S-components CbrT and FolT2, which interact with the same ECF module in *L. delbrueckii*, do not share significant sequence similarity (16% identical residues). Accordingly, the structures show pronounced differences (rmsd 3.1 Å), although the overall folds are the same. Particularly, differences in loop 3 and loop 5 cause alterations of the protein surfaces that interact with the membrane domain of EcfT (Figure 5c and d). Therefore, tight association of the different S-components with the same ECF module requires the conformational adaptations in the membrane domain of the interacting EcfT subunits (Figure 5c and d).

CbrT is in a ‘toppled’ orientation in the ECF-CbrT complex with TMS 1, 2, 3, and 4 oriented almost parallel to the membrane plane. Although OH-Cbl was added in excess to the crystallization condition, the substrate was not bound. The *apo* state of the toppled S-component was observed before in other ECF-type transporters and likely represents the inward state after substrate release (22, 33–35). The absence of substrate is in agreement with the proposed transport model, in that the inward oriented *apo* protein is a low affinity state (22). It has been hypothesized that this state precedes ATP hydrolysis, which leads to release of the S-component component from the ECF module, and reorientation of the S-component in the membrane, which brings the substrate binding site to the extracellular side (22).

**Figure 5:** Comparison of the structures of ECF-CbrT and ECF-FolT from *Lactobacillus delbrueckii*. a) Cartoon representation of ECF-CbrT from the perspective of the plane of the membrane. Cytoplasmic ATPases, EcfA and EcfA’, are colored in red, EcfT in cyan and CbrT in yellow. b) Structural differences between the membrane domains of EcfT. The structures of ECF-CbrT (cyan) and ECF-FolT2 (green) from *L. delbrueckii* were superimposed by structural alignment of the ATPase units. Pro71 of EcfT is represented in sticks. c) and d) Surface representation of CbrT (c, yellow) and FolT (d, orange) interacting with EcfT (cartoon representation colored in grey) with loop 3 of the S-components colored in blue. A dashed line highlights the movement of the transmembrane helix 3 of EcfT. e) and f) Loop 3 obstructs access to the substrate binding cavity in CbrT but not in FolT2. e) Slice-through of CbrT in surface representation, viewed from the plane of the membrane. Loop 3 is colored in blue. The ECF module has been omitted for clarity. f) Same slice through representation like in e) but for FolT2.
We hypothesize that the binding site for Cbl is located in a large cavity observed in the CbrT structure. The location of the cavity matches the position of the substrate-binding sites in the structurally characterized S-components in the substrate-bound state (21, 22, 32). In contrast to ECF-FolT2 (22), and structurally characterized ECF complexes from other organisms (33–35), the binding cavity in ECF-CbrT is largely occluded, and not accessible from the cytosol. The occlusion is caused mainly by the position of loop 3, which obstructs access to the cavity in ECF-CbrT. We speculate that occlusion of the empty binding site after cytoplasmic release of the substrate may be required for the subsequent reorientation of the S-component, upon release from the ECF module.

Discussion

Comparative genomics studies have identified a wide range of ECF transporter families along with their putative substrate specificities mediated by their S-components (15). Based on its genetic organization and the lack of a BtuCD-F transporter homolog in the Lactobacillales genomes, CbrT was predicted to be a vitamin B12-specific S-component (15). In L. delbrueckii, which was shown to be auxotrophic for vitamin B12 (36), CbrT occurs within the nrdJ–cbrS–cbrT–pduO gene cluster. This genetic organization strongly implies an involvement of the substrate binding protein CbrT in Cbl uptake: First, nrdJ is annotated as an Ado-Cbl dependent ribonucleotide reductase requiring the vitamin as a cofactor. Second, PduO is a cobalamin adenosyltransferase, which converts Cbl into Ado-Cbl making it accessible for NrdJ. We showed that ECF-CbrT not only transport Cbl but also mediates uptake of Cbi (Figure 3a). Therefore, L. delbrueckii is expected to have the genetic repertoire to synthesize Cbl using Cbi as a precursor. However, we could not find homologs of the enzymes CobS, CobU and CobT, which are known to convert Cbi into cobalamin in E. coli (29). Possibly other proteins might functionally compensate for their absence, which was shown for thiamin kinase YcfN, which could replace CobU in Salmonella typhimurium (37). However, it is also possible that L. delbrueckii CbrT binds Cbi in a futile manner, in which case Cbi would be transported but not used as a substrate for any enzyme. Finally, predicted CbrT homologs in Lactobacillales display a high degree of sequence identity, ranging from 25% to 60%
compared to \textit{L. delbrueckii} CbrT. Thus, we hypothesize that these CbrT homologs share the same function and represent substrate binding proteins for both Cbl and Cbi.

Our results show that ECF-CbrT is a new vitamin B12 transporter that is able to restore Cbl-Our results show that ECF-CbrT is a new vitamin B12 transporter that is able to restore Cbl- and Cbi-dependent growth in \textit{E. coli} ΔFEC (Figure 2c). Further characterization using uptake experiments with the purified ECF-CbrT complex (Figure 3a) and binding studies on CbrT (Figure 4) show that the transporter is promiscuous towards the β-ligand of Cbl and also accepts Cbi as substrate (Figure 3b). A similar behavior has also been observed for BtuCDF (12).

All three naturally occurring Cbl variants, OH-Cbl, Ado-Cbl and Met-Cbl, inhibit uptake of radiolabeled CN-Cbl (Figure 3b). Whereas almost full inhibition was observed by a 250-fold excess of OH-Cbl, CN-Cbl and Met-Cbl, Ado-Cbl inhibited only to 25%. This might be due to its bulkier β-ligand and a consequent steric hindrance. Nonetheless, the assay shows that ECF-CbrT is promiscuous towards the β-axial ligand of vitamin B12. Generally, poly-specificity to Cbl (and Cbl-derivatives, see below) and Cbi seems to be an inherent feature of vitamin B12 binding proteins, which is the case for BtuF, human Cbl-carriers and also CbrT (12, 38). In the human Cbl-carriers that share a similar promiscuity, the β-ligand side of the bound substrate is partially solvent exposed (39–41). In CbrT, the broader substrate specificity might be related to flexibility of loops 1 and 3 that are the gates of CbrT and would make contact with the varying β-axial ligands.

In other ECF-transporters, the S-components exhibit remarkable high affinities toward their respective substrates with $K_D$ values in the low nanomolar range (16). ITC measurements with CbrT in the absence of the ECF module also show high affinity binding with CN-Cbl, OH-Cbl, Met-Cbl, and Cbi (Figure 4b, c, and e). The slightly lower affinity for Cbi (4-fold) is probably due to the lack of the α-ligand (Figure 1 and Suppl. Figure 1) that leads to fewer possible protein-substrate interactions.

Strikingly, the affinity binding constants for CN-Cbl and Cbi are in the same range as the respective affinities determined for BtuF (Cbl 9.1 nM and Cbi of 40 nM) (12), which might imply that Cbl-transporters evolved to acquire the substrates with similar efficiency. Together with our $v_{max}$
and $K_M$ (Suppl. Figure 2) determination, we additionally show that the rate limiting step is substrate translocation, which means that the observed affinities are probably optimized for efficient substrate scavenging, followed by a slow translocation step. Human carriers achieve even higher affinities for Cbl (in the sub-picomolar range (38), but in these cases the off rate is practically zero and substrate release requires proteolysis, which is not the mechanism of (ABC) transporters.

Although it was already known for a long time that a plethora of prokaryotic vitamin B12 uptake systems must exist, only the BtuCDF complex had been extensively characterized. This is somewhat surprising, considering the potential relevance of bacterial vitamin B12 transport for pharmaceutical applications. For instance, given the increase in antibiotic resistance and its serious threat to public health (42), it is imperative to find and characterize novel protein targets for drug design. Several pathogenic bacteria, such as *Streptococcus pyogenes* and *Clostridium tetani*, carry a *cbrT* gene, lack a BtuCDF homolog, and are Cbl-auxotrophs, which makes them strictly dependent on dedicated transporters to scavenge either vitamin B12 or its precursors from the environment. Because humans use endocytosis to take up Cbl (43), Cbl-specific prokaryotic transporters are potential drug targets for vitamin B12 auxotrophic pathogens.

**Materials and Methods**

*Molecular Methods*

For expression, CbrT (LDB_RS00385) was amplified by means of polymerase chain reaction (PCR) using *L. delbrueckii* subsp. *bulgaricus* genomic DNA as a template. For expression of the entire complex, CbrT was inserted into the second multiple cloning site of p2BAD_ECF with *XbaI* and *XhoI* restriction sites (22). For expression of solitary CbrT, the gene was inserted with a C-terminal 8His-tag into pBAD24 using *NcoI* and *HindIII* restriction sites (44). A single glycine (Gly2) was introduced to be in frame with the start-codon of the *NcoI* restriction site, which is not present in the full complex. All primers used are listed in Suppl. Table 2 and all sequences were checked for correctness by sequencing.
**Expression and membrane vesicle preparation**

ECF-CbrT was expressed as described previously (22) with the following adaptations: Plain Luria Miller broth (LB) medium was used and the growth temperature was kept constant at 37°C throughout. After three hours of expression, the cells were harvested by centrifugation (20 min, 7,446×g, 4°C), and resuspended in 50 mM KPi, pH 7.5. Cells were either immediately used for membrane vesicles preparation or the resuspended cells were flash frozen in liquid nitrogen and stored at -80°C until use. Membrane vesicles were prepared as previously described (22).

**ECF-CbrT Purification**

Crude membrane vesicles containing ECF-CbrT were solubilized in buffer A (50 mM KPi, pH 7.5, 300 mM NaCl, 10% glycerol, 1% (w/v) n-dodecyl-β-D-maltopyranoside (DDM, Anatrace) for 45 min at 4°C under constant shaking. Insolubilized material was removed by centrifugation (35 min, 287 000 × g, 4°C), the supernatant was loaded on a BioRad PolyPrep column containing 0.5 mL Ni²⁺-sepharose bed volume (GE healthcare), pre-equilibrated with 20 column volumes (CV) buffer B (50 mM KPi, pH 7.5, 300 mM NaCl, 10% glycerol) and allowed to incubate for one hour at 4°C under constant movement. Unbound protein was allowed to flow through and the column was washed with 20 CV of buffer C (50 mM KPi, pH 7.5, 300 mM NaCl, 10% glycerol, 50 mM imidazole, 0.05% DDM). ECF-CbrT was eluted with buffer D (50 mM KPi, pH 7.5, 300 mM NaCl, 10% glycerol, 500 mM imidazole, 0.05% (w/v) DDM) in three fractions of 0.4 ml, 0.75 ml and 0.5 ml, respectively. ECF-CbrT eluted mostly in the second elution fraction that was loaded on a Superdex 200 Increase 10/300 gel filtration column (GE Healthcare) that was equilibrated with buffer E (50 mM HEPES pH 8.0, 150 mM NaCl, 0.05% DDM). For crystallization, ECF-CbrT was purified following the same protocol but buffers A to D contained 1% DDM. Buffers A to D were supplemented with 0.5 mM hydroxyl-cobalamin (OH-Cbl, Sigma Aldrich) and buffer E contained 10 μM OH-Cbl. For all experiments, the peak fractions were collected, combined and either used directly for reconstitution or concentrated in a Vivaspin disposable ultrafiltration device with a molecular weight cut-off of 30 kDa (Sartorius Stedim Biotech SA) to a final concentration of 6 mg ml⁻¹.
Construction of the E. coli ΔFEC strain

The E. coli strains JW0154 (ΔbtuF::KmR) and JW3805 (ΔmetE::KmR) from the Keio collection (23) were purchased from the Coli Genetic Stock Center, Yale. E. coli JW0154 (ΔbtuF::KmR) was used as the basis for constructing E. coli ΔFEC. The kanamycin resistance cassette of JW0154 was removed using the FLP recombinase as described before (24), resulting in E. coli ΔF. The metE::KmR locus from JW3805 was introduced in E. coli ΔF using P1-mediated generalized transduction as described (25, 45), resulting in E. coli ΔFE::KmR. The kanamycin cassette was removed using the FLP recombinase, resulting in E. coli ΔFE. The ΔbtuC::KmR locus of JW1701 was introduced in E. coli ΔFE using P1-mediated generalized transduction, resulting in E. coli ΔFEC. Colony PCRs based on three primer pairs (buF-locus, 5’-atggctaagtcactgttcagg-3’ & 5’-ctaatctacctgtgaaagcgc-3’; butC-locus, 5’-atgctgacacttgcccgc-3’ & 5’-ctaacgtcctgcttttaaca-3’; metE-locus, 5’-atgacaatattgaatcacaccctcg-3’ & 5’-ttacccccgacgcaagttc-3’) were used to verify KmR-insertions, the FLP-recombinase-mediated removal of KmR-markers and the absence of any genomic duplications resulting in the presence of any wild-type metE, butC and butF loci.

Growth assay with E. coli ΔFEC strains

The strains carrying various expression vectors were grown overnight at 37°C on LB-agar plates supplemented with 25 μg ml⁻¹ kanamycin and 100 μg ml⁻¹ ampicillin. The composition of the M9-based (47.7 mM Na₂HPO₄·12H₂O, 17.2 mM KH₂PO₄, 18.7 mM NH₄Cl, 8.6 mM NaCl) minimal medium was supplemented with 0.4% glycerol, 2 mM MgSO₄, 0.1 mM CaCl₂, 100 μg ml⁻¹ L-arginine, 25 μg ml⁻¹ kanamycin and 100 μg ml⁻¹ ampicillin. A single colony was picked and used to inoculate a 3 ml to 6 ml liquid pre-culture supplemented with 0.00001% L-arabinose (Sigma-Aldrich). The pre-culture was grown ~24 hours at 37°C, shaking in tubes with gas-permeable lids (Cellstar). The main cultures were inoculated in a 1:500 inoculation ratio. The main culture had a volume of 200 μl and was supplemented with 0.00001% L-arabinose (Sigma-Aldrich) and either 50 μg ml⁻¹ L-methionine, 1 nM dicyano-cobinamide (Sigma Aldrich), or 1 nM cyano-cobalamin (Acros Organics). The medium was added to a sterile 96 well-plate (Cellstar). The 96-well plate was sealed with a sterile and gas-permeable foil (BreatheEasy, Diversified
Biotech). The cultures were grown for 1000 minutes in a BioTek Power Wave 340 plate reader at 37°C, shaking. The OD$_{600}$ was measured every five minutes at 600 nm. All experiments were conducted as technical triplicates from biological triplicate. To obtain lag-times the averaged growth curves were fitted with the Gompertz-fit in Origin 8 and further analyzed as described (28).

**Crystallization and structure determination**

Initial crystallization conditions for ECF-CbrT were screened at 4 °C using commercial sparse-matrix crystallization screens in a sitting-drop setup and a Mosquito robot (TTP Labtech, UK). Initial crystals were found in the B11 condition (0.2 M KCl, 0.1 M Sodium citrate pH 5.5, 37% (v/v) Pentaerythritol propoxylate (5/4 PO/OH) of the MemGold1 HT-96 screen (Molecular Dimensions, UK) that diffracted up to 7.5 Å resolution. Using this condition as a starting point and the detergent (HR2-408) screen (Hampton Research, USA), an optimized condition could be found and contained the detergent ANAPOE®-C$_{12}$E$_{10}$ (Polyoxyethylene(10)dodecyl ether, Hampton Research) as an additive, which yielded crystals diffracting up to 3.4 Å resolution.

X-ray diffraction data was collected from cooled (100 K) single crystals at synchrotron beam lines at the Swiss Light Source (SLS, beamline PX1), Switzerland. The crystals of apo ECF–CbrT belong to space group P1 (unit cell parameters: a=85.47, b=92.86, c=105.51, α=72.568, β=66.274, γ=62.893).

To correct for anisotropy, the dataset was treated with the diffraction anisotropy server prior to further processing (46). Data were processed with XDS (47) and scaled with Xscale (48). Data collection statistics are summarized in **Suppl. Table 1**. The structure of the ECF-CbrT complex was solved by molecular replacement with PHASER MR (49) using the apo ECF-FolT2 structure of *L. delbrueckii* (22) (PDB code 5JSZ) as a search model. For model completion, several cycles of model building with COOT (50) and refinement with PHENIX (51) were performed. The Ramachandran statistics are 72.32% for favored regions, 26.64% for allowed regions and 1.05% for outliers. All structural figures in the main text were prepared with open-source version of pymol (https://sourceforge.net/projects/pymol/).
**Preparation of radiolabeled cobinamide from radiolabeled cobalamin**

The required amount of cyano-cobalamin (radiolabeled and unlabeled) was mixed in a 1:1 (v/v) ratio with 70% perchloric acid (Sigma-Aldrich) and incubated for ten minutes at 70°C. To quench the reaction and prevent damage to the substrate, the resulting cobinamide substrate was added to buffer G (as described above), which was additionally supplemented with 5 M NaOH to restore the pH back to 7.5.

**Radiolabeled vitamin B12 transport assay**

Purified ECF-CbrT was reconstituted in proteoliposomes as described previously (52). Proteoliposomes were thawed and loaded with 5 mM MgSO\(_4\) or MgCl\(_2\) and 5 mM Na\(_2\)-ATP or Na\(_2\)-ADP through three freeze-thaw cycles. Loaded proteoliposomes were extruded nine times through a polycarbonate filter with a 400 nm pore-size (Avestin), pelleted by centrifugation (267,008 g, 35 minutes, 4°C) and resuspended in buffer F (50 mM KPi pH 7.5) to 2 μl/mg lipids. The uptake reaction was started by addition of concentrated and loaded proteoliposomes to buffer G (50 mM KPi pH 7.5, varying concentrations of \(^{57}\)Co-cyanocobalamin (150 to 300 μCi/mg, in 0,9% benzylalcohol, MP Biomedicals) in a 1:100 ratio. At elsewhere specified time points 200 μl samples were taken transferred into 2 ml ice cold buffer F and filtered over OE67 cellulose acetate filters (GE Healthcare) soaked in Buffer F supplemented with cyanocobalamin (Acros chemicals). The filter was washed with 2 ml ice cold buffer F and transport of radiolabeled substrate was counted in Perkin Elmer Packard Cobra II gamma counter. All uptake assays were performed at 30°C while stirring.

**Substrate-binding assay**

ITC measurements were performed using a NanoITC calorimeter (TA Instruments) at 25 °C. Membrane vesicles containing CbrT (200μl, 10 mg/ml in 50 mM Kpi, pH 7.5) were added to the NanoITC cell. Ligands were prepared in 50 mM KPi, pH 7.5 and titrated into the cell in 1ul injections with 140s between each injection. Membrane vesicles containing the full-complex ECF-CbrT that does not bind CN-Cbl (10 mg/ml in 50 mM Kpi, pH 7.5) were used as a negative control. Data were analyzed with the Nano Analyze Software.
**Data deposition**

The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 6FNP).

**Acknowledgments**

We thank Prof. Dr. A.J.M. Driessen for the use of the setup in the isotope lab and the beamline personnel of PXI at SLS for their technical support. This work was supported by grants from the Netherlands Organisation for Scientific Research (NWO Vici grant 865.11.001 to D.-J.S. and NWO Vidi grant 723.014.002 to A.G.), the São Paulo Research Foundation (BEPE fellowship 2015/26203-0 to C.T.P), the European Research Council (ERC starting grant 282083 to D.-J.S.), and the European Molecular Biology Organization (EMBO long-term fellowship ALTF 687-2015 to J.A.S. and EMBO short-term fellowship ASTF-382-2015 to S.R.).

**References**

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Supplementary Information

Supplementary tables

Suppl. table 1: Data collection, phasing and refinement statistics.

<table>
<thead>
<tr>
<th>ECF-CbrT</th>
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<td><strong>Data collection</strong></td>
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<tr>
<td>Space group</td>
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<tr>
<td><strong>Unit cell dimensions</strong></td>
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<tr>
<td>$a$, $b$, $c$ (Å)</td>
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<tr>
<td>$\alpha$, $\beta$, $\gamma$ (°)</td>
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<tr>
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<td>$R_{merge}$ (%)</td>
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<tr>
<td>$I/\sigma I$</td>
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<tr>
<td>Completeness (%)</td>
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<tr>
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<td><strong>R.m.s. deviations</strong></td>
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<td>Bond angles (Å)</td>
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\(^a\)Values in parentheses are for the highest-resolution shell.

Suppl. table 2: Primer list used in this study.

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<tr>
<td>Seq_rev</td>
<td>GCTGAAAAATCTTCTTCTCATCCG</td>
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Supplementary figures

Suppl. Figure 1: 3D structures of cobalamin and cobinamide. Cobalamin (green) and cobinamide (cyan) are shown in stick representation. The central cobalt ion is colored in grey, nitrogen atoms in blue, oxygen atoms in red and phosphate atoms in orange.
Suppl. Figure 2: Kinetics of cobalamin uptake by ECF-CbrT. Transport rates are shown as a function of the cobalamin concentration. Proteoliposomes were loaded with 5 mM Mg-ATP. Experiments were performed in triplicated and error bars show the standard deviation of the mean. The data was fitted with a Michaelis-Menten function to obtain the $K_M$ value of 2.1 ± 0.4 nM and the $V_{max}$ value of 0.06 ± 0.01 pmol mg$^{-1}$ s$^{-1}$. 
Suppl. Figure 3: Binding of Cbl-analogs to CbrT. ITC measurements of a) OH-Cbl and b) CH$_3$-Cbl binding to CbrT. The determined $K_D$ values were averaged from duplicate measurements and the error is the standard deviation of the mean. Fitting of single binding site models to the data is shown in c).