Conformational Change of a Tryptophan Residue in BtuF Facilitates Binding and Transport of Cobinamide by the Vitamin B12 Transporter BtuCD-F

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BtuCD-F is an ABC transporter that mediates cobalamin uptake into *Escherichia coli*. Early *in vivo* data suggested that BtuCD-F might also be involved in the uptake of cobinamide, a cobalamin precursor. However, neither was it demonstrated that BtuCD-F indeed transports cobinamide, nor was the structural basis of its recognition known. We synthesized radiolabeled cyano-cobinamide and demonstrated BtuCD-catalyzed *in vitro* transport, which was ATP- and BtuF-dependent. The crystal structure of cobinamide-bound BtuF revealed a conformational change of a tryptophan residue (W66) in the substrate binding cleft compared to the structure of cobalamin-bound BtuF. High-affinity binding of cobinamide was dependent on W66, because mutation to most other amino acids substantially reduced binding. The structures of three BtuF W66 mutants revealed that tight packing against bound cobinamide was only provided by tryptophan and phenylalanine, in line with the observed binding affinities. *In vitro* transport rates of cobinamide and cobalamin were not influenced by the substitutions of BtuF W66 under the experimental conditions, indicating that W66 has no critical role in the transport reaction. Our data present the molecular basis of the cobinamide versus cobalamin specificity of BtuCD-F and provide tools for *in vitro* cobinamide transport and binding assays.

ATP-binding cassette (ABC) transporters are a large family of integral membrane proteins and involved in nutrient uptake, drug extrusion, and lipid homeostasis. They use the energy of ATP binding and hydrolysis to power substrate transport across the lipid bilayer. Depending on the direction of transport, into the cytoplasm or out, they are classified as importers or exporters, respectively. Prokaryotic importers depend on external substrate binding proteins (SBPs) for delivery of the cargo to the membrane-embedded transporters. In the Gram-negative bacterium *E. coli*, BtuF is the periplasmic SBP that delivers cyanocobalamin (Cbl) to the ABC type II importer BtuCD. BtuCD-F has been studied extensively by structural and functional techniques and a detailed transport mechanism has been formulated to rationalize the coupling of nucleotide binding and hydrolysis to conformational changes facilitating active transport.

*E. coli* is unable to synthesize Cbl de novo. However, it expresses enzymes that facilitate its synthesis from the precursor cobinamide (Cbi), which lacks the 5,6-dimethylbenzimidazole (DMB) moiety and sugar-phosphate linker and is therefore smaller than Cbl (Figs 1A and 2C). Before the individual proteins (BtuC, BtuD, BtuF) were identified, *in vivo* transport data suggested that the *E. coli* uptake system specific for Cbl also transports Cbi. In line with this observation, recent *in vitro* binding data showed that *Thermotoga* species BtuF variants can bind not only Cbl, but also Cbi. The authors base their finding on changes in Tm values measured by differential scanning fluorimetry and shifts in the visible spectra upon ligand binding. They propose that *Thermotogales* species BtuCD-F transporters can import both Cbl as well as Cbi. Although the Cbl-bound BtuF structures revealed the
molecular organization of the substrate binding site at high resolution\textsuperscript{15,16}, it is not known whether and how \textit{E. coli} BtuF would recognize and bind Cbi in addition to Cbl. Also, Cbi transport by BtuCD-F was never demonstrated.

Figure 1. Generation of radioactive cobinamide and \textit{in vitro} transport by BtuCD. (A) Reaction scheme of $^{14}$CN-Cbi generation ($^{14}$C-labeled cyano-aquo-cob(III)iminamide). Radiolabeled cobinamide was generated in an oxidative reaction by addition of $^{14}$C-labeled KCN to aquo-cob(II)iminamide. The CN group in the product can be coordinated in the $\alpha$- or $\beta$-position of the corrin ring. (B) UV/Vis spectra of $^{14}$CN-Cbi generation. A 0.85 mol equivalent of $K^{14}$CN was titrated to 4.8 $\mu$mol aquo-cob(II)iminamide at 22°C. UV/Vis spectra of the reaction mix diluted to 47 $\mu$M cobinamide were recorded 30 to 60 min after each sequential addition of $K^{14}$CN and ddH$_2$O was used for the blank measurement. The final product cyano-aquo-cobinamide is represented with the black line and the educt aquo-cob(II)iminamide is shown in brown. Titration steps in between are shown with thin lines. (C) Schematic of \textit{in vitro} Cbi transport by BtuCD reconstituted into liposomes. Only the right-side-in oriented BtuCD molecules are accessible for BtuF and yield successful transport. The direction of substrate transport into the liposome lumen is indicated with a pink arrow. To keep a constant ATP concentration within the time course of the experiment, an ATP-regenerating system (A.R.S.) was incorporated into the liposome lumen. (D) Cbi transport activity of wild type BtuCD-F. Transport was measured under the following conditions: 4 mg/ml proteoliposomes (~0.5 $\mu$M total BtuCD), 1 $\mu$M BtuF, 15 $\mu$M cobinamide and 2 mM ATP. Unspecific radioligand binding to liposomes was determined by omission of ATP or BtuF. Shown are mean ± S.E.M. ($n=6$) of initial transport rates. For lines used to determine initial rates see Supplementary Figure 2.
We pursued structural and functional approaches to address these questions. We developed an in vitro transport assay and showed that BtuCD could indeed transport Cbi in an ATP- and BtuF-dependent manner. We also established an in vitro binding assay that allowed Cbi and Cbl binding to BtuF to be quantitated. We identified a single tryptophan residue (W66) in BtuF that adopts a distinct conformation depending on whether Cbi or Cbl is bound.

**Results**

**14CN-Cbi synthesis and in vitro transport.** To investigate in vitro Cbi transport by BtuCD, radiolabeled substrate was required, which is not commercially available. We exploited the fact that CN-Cbi (cyano-aquo-cobinamide) is a stable and inert species and synthesized 14CN-Cbi by an oxidative reaction using aquo-cob(II)inamide and 14C-labeled KCN as educts (Fig. 1A). A 0.85 mol equivalent of K14CN was titrated to aquo-cob(II)inamide, since a 1:1 ratio already leads to the formation of a small fraction of di-cyano-cobinamide. The 14CN-Cbi product formation was monitored by recording UV/Vis spectra, which clearly showed shifts of the absorption...
BtuF-dependent, as was observed for Cbl transport. The calculated capability in absolute rates depending on the proteoliposome batch. BtuCD-catalyzed Cbi transport was ATP- and BtuCD-F. was approximately 1.5-fold slower than that of Cbl transport. This makes Cbi a bona fide substrate of compared to wt, and a change to histidine or arginine reduced it more than 10-fold (Table 1). The largest change except for W66F (30-fold) compared to wt. The K_d of wt BtuF for Cbi was 40 ± 10 nM, and the affinity of the W66X mutants was lower except for W66F (30 ± 7 nM, Table 1). Substitution of W66 with tyrosine or leucine reduced the affinity 3-fold compared to wt, and a change to histidine or arginine reduced it more than 10-fold (Table 1). The largest change in affinity was observed for W66E.

Crystal structure of Cbi-bound BtuF. The crystal structure of mono-cyano-cobinamide bound BtuF (CN-Cbi-BtuF) was determined at 1.5 Å resolution by molecular replacement using Cbl-bound BtuF (PDB ID 1N4A) as a model (Fig. 2). Well-defined electron density in the difference map was observed for the CN group bound to the Co atom, facing the W66 side chain. Residues 217–232 in Cbi-bound BtuF were not visible in the electron density, indicating flexibility in this region, whereas this stretch forms an α-helix in Cbl-bound BtuF. It is not clear whether this is a crystallographic artefact or of biological relevance. Similarly high flexibility in this region was observed for the apo-structure of BtuF16.

Binding of Cbi and Cbl to BtuF W66 mutants. To investigate the role of W66 in substrate binding and transport, we generated various W66 mutations and analyzed in vitro substrate binding with a fluorescence based binding assay. The binding assay was performed with CN-Cbi or di-CN-Cbi and BtuF_W66 (Suppl. Figure 3). We found that binding of Cbl or Cbi quenches the fluorescence of the covalently attached fluorescein label, allowing the precise determination of bound substrate. Using this assay, we determined a dissociation constant K_d of Cbl and wt BtuF_W66 in the low nM range, as reported previously. No substantial changes in affinity for Cbl could be determined for W66 mutants (Fig. 3 and Table 1) except for mutations to charged side chains (arginine or glutamate), which caused a decrease in affinity of ~7-fold. In contrast, binding of Cbi to BtuF was strongly affected by mutations of W66. The K_d of wt BtuF for Cbi was 40 ± 10 nM, and the affinity of the W66X mutants was lower except for W66F (30 ± 7 nM, Table 1). Substitution of W66 with tyrosine or leucine reduced the affinity 3-fold compared to wt, and a change to histidine or arginine reduced it more than 10-fold (Table 1). The largest change in affinity was observed for W66E.
Supplementary Figure 4. The substrate binding assay is based on fluorescence quenching of fluorescein labeled BtuF (BtuFfluo) in Table 1.

We have developed a method to synthesize radiolabelled Cbi and have established an in vitro Discussion Cbl and Cbi transport by BtuCD with BtuF W66 mutants.

In vitro

Crystal structures of Cbi-bound BtuF mutants W66F, W66Y and W66L. To visualize the structural basis of the observed functional effects of W66 mutations, crystal structures of Cbi-bound BtuF containing the mutations W66F, W66Y or W66L were determined at similar resolutions as the wild type construct (Table 2). The refined wild type model with mutated W66A and deleted water molecules was used as a search model for molecular replacement. The structures of the mutants were similar to that of the wild type, and differences were only observed in the substrate-binding pocket (Fig. 4). The side chains of the mutated residues at position 66 could all be modeled, although the quality of the electron density maps was not as high as for the wild type. Electron density was visible for W66F in both protein chains of the asymmetric unit, but mainly in the 2Fo-Fc omit map. The W66F side chain was oriented similarly to that of the wild type tryptophan, which is in line with the similarly high Cbi binding affinities of these two BtuF variants. In contrast to W66 and W66F, the leucine side chain of the W66L variant was pointing out of the substrate-binding pocket, reminiscent of the W66 rotamer in the Cbi-bound BtuF structure. Clear density for the leucine side chain was observed in the Fo-Fc omit map for both BtuF W66L chains in the asymmetric unit. A stretch of continuous extra density was present near the corrin ring, where the tryptophan and phenylalanine side chains were found in the wild type or W66F variant of BtuF. This extra density likely originated from a bound buffer component and was modeled as a glycerol molecule that may have entered this cavity during purification or crystal cryoprotection. In the structure of the W66Y variant, density both at the α- and β-positions, and we therefore modeled a cyanide group on both sides, each with an occupancy of 0.5. Thus, the BtuF substrate binding site does not seem to have a preference for the α- or β-position, although cyanide could not be assigned to one side of the corrin ring consistently. The corrin ring nevertheless was always bound with the α-side facing the N-lobe and the β-side facing the C-lobe of BtuF.

In vitro Cbl and Cbi transport by BtuCD with BtuF W66 mutants. Substrate transport was measured using commercially available 57Co-Cbl and in-house synthesized 14CN-Cbi as described above. To examine the role of W66 in substrate delivery and transport, three mutants with impaired Cbi binding (W66A, W66R, and W66E) and one with high binding affinity (W66F) were used for transport assays. Despite having lower Cbi binding affinities, Cbi transport was hardly affected by W66X substitution (Fig. 5). This finding indicates that under the conditions used, W66 is important for high affinity Cbi binding, but not for substrate delivery or transport.

Discussion

We have developed a method to synthesize radiolabelled Cbi and have established an in vitro transport assay that confirms that the E. coli BtuCD-F binds and transports the Cbl precursor Cbi. Wild type BtuCD-F transports Cbi

| E. coli BtuF | K0 of Cbl, nM | K0 of Cbi, nM or μM |
|-------------|--------------|---------------------|
| Wild type   | 9.1 ± 2.6    | 40 ± 10 nM          |
| W66F        | 8.0 ± 1.9    | 30 ± 7 nM           |
| W66Y        | 5.7 ± 1.7    | 0.15 ± 0.03 μM      |
| W66L        | 15 ± 2       | 0.12 ± 0.02 μM      |
| W66H        | 5.5 ± 1.7    | 0.5 ± 0.11 μM       |
| W66R        | 68 ± 13      | 0.8 ± 0.2 μM        |
| W66A        | 14 ± 4       | 1.8 ± 0.8 μM        |
| W66E        | 57 ± 15      | 2.9 ± 0.8 μM        |

Table 1. Dissociation constant K0 values of Cbl and Cbi binding to wild type BtuF and W66 mutants. The substrate binding assay is based on fluorescence quenching of fluorescein labeled BtuF (BtuFfluo) in a substrate concentration dependent manner. BtuFfluo was used at 5 nM and substrate concentration was varied from 0.02 nM to 33 μM. Note that K0 values are represented in nM or μM and indicated are mean and S.E.M. (n = 12, for W66R n = 9). Example curves used to determine the K0 values are shown in Fig. 3 and Supplementary Figure 4.
approximately 1.5-fold slower than Cbl, which illustrates that the size of the substrate does not inversely correlate with the rate of transport. Instead, interaction between the substrate and the translocation pathway between the BtuC subunits might affect the transport rate.

To elucidate the molecular basis of the Cbi versus Cbl specificity of BtuF, we determined the X-ray structure of Cbi-bound BtuF. A single residue, W66, was found to have a pivotal role. This residue is located in the binding site and appears to replace and thus compensate for the missing DMB group when Cbi is bound. It does so by changing its side chain rotamer to point towards the inside of the substrate binding pocket. A mutagenesis study of W66 showed that the presence of a big hydrophobic and aromatic side chain at this position is crucial for high affinity Cbi binding. In contrast, Cbl binding was much less influenced by the chemical properties of this residue, which is in line with the observed structure of W66 in Cbl-bound BtuF, where the indol moiety points away from the substrate binding pocket. Intriguingly, MD simulations had earlier suggested that the BtuF segment including W66 may act as a gate in the substrate binding and unbinding process of BtuF\textsuperscript{17}. Our observations are in agreement with this interpretation, but single out W66 as the key residue adapting its conformation to facilitate Cbi binding.

Despite the lower binding affinities of certain W66 mutants for Cbi, substrate transport was not influenced by W66 substitution. This indicates that W66 is not involved in substrate delivery during a productive transport cycle. The transport assays were performed with a substrate concentration at which the BtuF binding pocket was fully occupied. This was crucial to prevent the binding of apo-BtuF to BtuCD, which would reduce the observed

| Data collection | BtuF-Cbi | BtuF\textsubscript{W66F}-Cbi | BtuF\textsubscript{W66Y}-Cbi | BtuF\textsubscript{W66L}-Cbi |
|----------------|---------|-----------------|----------------|----------------|
| Wavelength (Å) | 1.0000  | 0.9779          | 0.9779         | 0.9779         |
| Resolution range (Å) | 26.7–1.5 | 19.7–1.7 | 26.7–1.6 | 19.7–1.5 |
| Space group | C2      | C2              | C2             | C2             |
| Unit cell dimensions | 138.15 90.56 50.84 | 136.56 90.67 50.81 | 139.83 90.8 51.07 | 136.28 90.46 50.81 |
| a, b, c (Å) | 90 110.90 90 90 110.74 90 | 90 110.87 90 90 110.74 90 | 90 110.74 90 90 110.74 90 | 90 110.74 90 90 110.74 90 |
| Total reflections | 316217 (29743) | 188811 (18658) | 208631 (20239) | 271108 (26105) |
| Unique reflections | 89627 (8616) | 61184 (6145) | 73585 (7648) | 89398 (8823) |
| Multiplicity | 3.5 (3.4) | 3.1 (3.0) | 2.8 (2.8) | 3.0 (2.9) |
| Completeness (%) | 96 (93) | 96 (97) | 99 (95) | 97 (97) |
| Mean I/σ(I) | 10.21 (1.26) | 10.89 (1.39) | 8.77 (1.56) | 11.00 (1.53) |
| Wilson B-factor (Å\textsuperscript{2}) | 23.05 | 25.01 | 25.55 | 20.22 |
| Rmerge (%) | 5.9 (84.9) | 7.2 (81.6) | 6.4 (66.5) | 6.0 (61.0) |
| Rfree (5% of data) (%) | 7.0 (100.4) | 8.7 (99.8) | 7.8 (81.8) | 7.3 (74.3) |
| CC (%) | 99.8 (74.8) | 99.7 (64.7) | 99.7 (65.3) | 99.8 (59.3) |

Table 2. Crystallographic table with data collection and refinement statistics. Highest-resolution shell values are shown in parentheses. The correlation coefficient is abbreviated CC\textsuperscript{43}.
Figure 4. Stereo view of the substrate binding sites of Cbi-bound BtuF mutants in comparison to the wild type. Displayed are residue W66 in wild type BtuF (A) and the corresponding mutants W66F (B), W66Y (C) and W66L (D). Cobinamide was bound in all 4 crystal structures and was used as reference point for model alignment. In W66F the phenylalanine rotamer is as in the wild type structure facing the inside of the cavity. In W66Y as well as in W66L, the side chain is facing outside of the cavity. A glycerol molecule shown in yellow is occupying the space instead. Glycerol was used during purification and for cryoprotection, but was not present in the crystallization condition. The position of bound cyanide varied between the two protein chains in the asymmetric unit. For W66, W66F and W66Y chain A is shown and for W66L chain B. For the mutants W66Y and W66L only the cyanide group is shown, because no electron density was visible for the second axial ligand. The two potential axial ligands, cyanide and a water molecule, are represented in stick and red sphere, respectively. CN-Cbi is illustrated as stick representation (carbon, mangenta; nitrogen, blue; oxygen, red; Co, light brown). Displayed is the difference density of an omit map (Fo-Fc contoured at ±3.0σ) calculated by Phenix and using a model in which the occupancy of the side chain and Co were set to zero. See result section for more detailed description. PDB IDs of the models are 5M29 (BtuF-Cbi), 5M2Q (BtuF_{W66F}-Cbi), 5M34 (BtuF_{W66Y}-Cbi) and 5M3B (BtuF_{W66L}-Cbi).
transport rate below the detection limit. Because of this experimental limitation, it was impossible to perform transport assays at lower Cbi concentrations. Nevertheless, our data suggest that accepting Cbi or Cbl as substrates requires a conformational change in BtuF, whereas the transmembrane BtuC2 component appears inert to the chemical differences of Cbi versus Cbl.

Analogous to BtuF, FhuD is another class III periplasmic substrate binding protein18–20, for which multiple substrate specificity has been described. FhuD is part of the iron-siderophore (ferric hydroxamate) transport system responsible for substrate delivery to the ABC transporter FhuBC. The crystal structure of gallichrome-bound FhuD identified R84 and Y106 as the two key residues involved in direct hydrogen bonding with the three hydroxamate moieties of the ligand21. The main interaction of gallichrome with FhuD is mediated by the hydroxamate groups and a large part of the ligand is solvent exposed. This explains why other hydroxamates such as coprogen, Desferal, or the antibiotic albomycin are also bound by FhuD. The crystal structures revealed that albomycin is bound in a similar way to gallichrome, but coprogen binding involves Y275 for hydrogen bonding to the hydroxamate moiety22. The most drastic change in coprogen-bound FhuD is the reorientation of W217 to allow the insertion of part of the ligand, reminiscent of W66 in BtuF.

Multiple substrate specificity has also been observed for other periplasmic substrate binding proteins. For example, DEBP (L-aspartate/L-glutamate binding protein) from Shigella flexneri23 can bind both aspartate and glutamate while simultaneously discriminating against glutamine and asparagine. In this case, the side chain carboxylate of the ligand interacts with the side chains of two arginines, a serine and a histidine in the substrate binding site. It was speculated that conformational rearrangements of these residues are involved in adapting to the smaller substrate aspartate, which is analogous to the conformational rearrangement we observed in W66 of BtuF when bound to Cbi.

Poly-specificity has also been reported for components of the eukaryotic Cbl uptake system. Haptocorrin (HC), a human corrinoid binding protein, binds Cbl, Cbi, and other Cbl analogs with similarly high affinities24. Three bulky residues in the substrate binding site (an arginine, a tryptophan and a tyrosine) are important for Cbi binding to HC and indirectly compensate for the “missing” DMB moiety24,25. Specifically, the Cα atom of R357 moves ~2 Å towards the corrin ring and its side chain reorients towards the e-propionamide group of Cbi, thus stabilizing it in an alternate conformation via hydrogen bonding. This occurs on the same side where the DMB of bound Cbl would be located and has a space filling function similar to W66 in BtuF.

The substrate specificity of the maltose/maltodextrin uptake system, MalEFGK2, was also extensively studied. MalE, the periplasmic SBP, can bind maltose, maltotriose, and larger glycans up to maltodecapentaose (malto-dextrin with n = 15), as well as cyclic glycans, all with similarly high affinities26,27. Structures of MalE bound to maltose, maltotriose and maltotetraose revealed that the solvent accessible amount of the substrate increases with substrate size28. The first three glucose units are buried in the substrate binding groove, while additional units are exposed to the surface. Nevertheless, the binding specificity of MalE does not fully correlate with the transport rates. MalFGK2 translocates glycans up to maltoheptaose, but the transport rates decrease with increasing substrate size. Moreover, cyclic substrates are not transported even though they are bound by MalE26,28. In this case, both the SBP and the transporter contribute to substrate specificity.

The glutamine/asparagine transporter GlnPQ has a different approach to enable dual substrate specificity. Instead of using a single, poly-specific SBP, two structurally distinct substrate binding domains (SBD1 and SBD2) are tandem-linked to the N-terminus of one transmembrane domain29. SBD2 has a much higher affinity for glutamine than SBD1, which in addition to glutamine binds asparagine. Three non-conserved residues are responsible for the different affinities for glutamine, although they are not directly involved in substrate binding. They
presumably have a crucial role in full closure of the substrate binding site and thus lead to tighter ligand binding\(^\text{32}\). Furthermore, the size of the amino acid substrate itself affects opening and closure of the substrate binding site.

It is currently unknown whether other Cbl analogues can serve as substrates for BtuCD-F. However, our findings not only establish Cbi as a bona fide substrate of BtuCD-F, but offer opportunities to investigate the translocation pathway of this transporter by structural and biochemical studies, because not only can the two axial positions of the corrin ring of Cbi be modified with probes, but Cbi itself is smaller and can interact with histidine residues that might trap the substrates in the translocation pathway of BtuCD.

**Materials and Methods**

**Generation of radiolabeled cobinamide.** Cob(II)aminic acid was synthesized using a previously described method\(^\text{33}\) and \(^{14}\)C-cyanide-labeled through an oxidative reaction with \(^{14}\)CN (Perkin Elmer NCI079HH01MC, 1 mCi) generating \(^{14}\)C-cyanoadquo-cobinamide. The mono-cyano form was preferred over the di-cyano form due to higher stability in aqueous solution and thus facilitating unambiguous quantification. A 0.85 mol equivalent of \(^{14}\)CN was added in multiple steps to an aqueous solution containing 4.8 \(\mu\)mol aquo-cob(II)aminic acid at \(22^\circ\)C. The conversion of cob(II)aminic into \(^{14}\)C-cyanoaquocobinamide was followed by recording the UV/Vis spectrum and monitoring TLC migration pattern. The mixture was applied to reverse phase (C18) chromatography, equilibrated and washed with ddH\(_2\)O, and products were eluted with solvents of decreasing polarity: 5:5 MeOH:H\(_2\)O, 7:3 MeOH:H\(_2\)O, 100% MeOH, 100% EtOH, and 100% 2-Propanol. Fractions were collected and the \(^{14}\)C-labeled substrate concentration was determined by using \(^{14}\)C-CN stock dilutions as calibration curve. Products in the 5:5 MeOH:H\(_2\)O eluent were used for transport assays and stored at \(\sim -20^\circ\)C.

**BtuCD purification and reconstitution into liposomes.** BtuCD was expressed, purified and reconstituted as described previously with some modifications including His6-tag cleavage and periplasmic preparation in case of BtuF\(^\text{9,10}\). Additional purification steps using preparative size exclusion chromatography were performed as described previously for ABC type II importer HmuUV with minor modifications\(^\text{34}\). Briefly, liposome-reconstituted BtuCD was incubated with 1 \(\mu\)M fluorescently labeled BtuF (BtuF\(_{\text{fluo}}\)) for 30 min at RT followed by a spin to remove excess BtuF\(_{\text{fluo}}\). The pellet was washed and resuspended at 1.25 mg/ml liposome concentration. The amount of BtuF\(_{\text{fluo}}\) associated with the liposomes was determined by measuring the fluorescence signal at an excitation wavelength of 485 nm and an emission wavelength of 516 nm. Unspecific BtuF\(_{\text{fluo}}\) interaction with liposomes was measured using empty liposomes.

**Substrate transport assay with liposome reconstituted BtuCD.** For the substrate transport assays radiolabeled \(^{14}\)C-cyanoaquocobinamide (\(^{14}\)CN-Cbi, labeled in house) or \(^{57}\)Co-cyanocobalamin (\(^{57}\)Co-Cbl, MP Biomedicals 06B-430000, 10.5 \(\mu\)Ci) was used. Prior to reaction start substrate-loaded BtuF and BtuCD containing liposomes were incubated separately for 30 min to equilibrate to \(22^\circ\)C. To start the transport reaction substrate-loaded BtuF was added to the BtuCD containing liposomes with final concentrations of 15 \(\mu\)M substrate, 1 \(\mu\)M BtuF and 4 mg/ml liposomes (\(-0.5\) \(\mu\)M BtuCD). An ATP-regenerating system was incorporated into the liposome lumen to keep a constant ATP concentration within the time course of the experiment. Initial luminal ATP concentration was 2 mM. The transport reaction was stopped by diluting 50 \(\mu\)l samples in 300 \(\mu\)l ice cold stop buffer with the following composition: 20 mM Tris 7.5, 200 mM NaCl, 5 mM MgCl\(_2\), 8% w/v PEG6000 and 100 \(\mu\)M unlabeled Cbl. Samples were transferred to a manifold filtration system (Millipore MSFBN6B) and washed with 2 \(\times\) 200 \(\mu\)l cold stop buffer. The radioactivity trapped on the filters was measured either by a \(\beta\) counter using LCS in case of \(^{14}\)CN-Cbi or a \(\gamma\) counter in case of \(^{57}\)Co-Cbl. Initial transport rates were determined by using linear regression in PrismGraphpad.

**Purification and crystallization of Cbi-bound BtuF.** BtuF purification was performed as described previously with some modifications including His6-tag cleavage and periplasmic preparation in case of BtuF W66X mutants\(^\text{9,10}\). Additional purification steps using preparative size exclusion chromatography were performed to obtain purer sample. Final buffer composition was 10 mM Tris pH 8 and 100 mM NaCl. For crystallization purposes BtuF was concentrated in the presence of 0.2 mM di-cyano-cobinamide (Sigma C3021). Protein was supplemented with \(-1\) mM di-cyano-cobinamide prior to setting up crystallization trays at \(-20^\circ\)C. The crystallization experiment was performed by sitting drop vapor diffusion technique and incubated at \(20^\circ\)C. Depending on the pH of the reservoir condition, HEPES pH 7 or Tris pH 8.2 and higher, the crystallization drops appeared orange-red or purple corresponding to the mono-cyano- or di-cyano-form of cobinamide, respectively. This observation was made despite the fact that BtuF was co-crystallized with di-cyano-cobinamide when setting up the crystallization drops. Initial wild type crystals were obtained after 3 days, while the mutant crystals grew within 1–2 weeks in optimized screens. Initial crystallization conditions were from PEG/ION Hampton screen condition H11 (1% w/v Tryptone, 50 mM HEPES sodium pH 7.0 and 12% w/v Polyethylene glycol 3350) and were optimized in 24-well plates. Orange-red rod-shaped crystals grew to \(-300\) microns in size and were cryoprotected by stepwise increase of glycerol to 25% w/v. Mono-cyano-cobinamide was bound in the crystal structures corresponding to the drop and crystal colors.
Protein concentration was determined by Bradford assay using BSA for calibration and the labeling efficiency removed by desalting with PD10 columns and the protein was further purified by size exclusion chromatography.

80 °C until the day of experiment.

emission of fluorescein attached to BtuF (BtuFfluo). A similar approach has been used to determine the affinity of PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

refinement and model building were performed with Phenix and Coot37,38. Additional refinement steps were W66X mutants were obtained using the wild type structure with W66A and deleted waters. Iterative cycles of refinement and model building were performed with Phenix and Coot57,38. Additional refinement steps were performed with Refmac5 in the CCP4 program suite. PDB file modifications like removing Hydrogens, ANISO records or alternative conformations were performed using the CCP4 program suite39. Final figures were prepared using PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

Purification of BtuF for fluorescein labeling (BtuFfluo) and substrate transport assays. For binding assays BtuF was labeled at a cysteine residue in the backbone helix with fluorescein-5-maleimide (Thermo scientific 62245) as described previously11. In brief, 50 to 100 μM BtuF Q145C (Invitrogen QuickChange) was labeled with a 10-fold molar excess of fluorescein-5-maleimide (BtuFfluo). The reaction was run over night at 4 °C in the presence of 1 mM TCEP to prevent oxidation and stopped with 5 mM β-mercaptoethanol. Excess label was removed by desalting with PD10 columns and the protein was further purified by size exclusion chromatography. Protein concentration was determined by Bradford assay using BSA for calibration and the labeling efficiency was determined by measuring the absorbance at 494 nm. The labeled protein was flash frozen in liquid nitrogen and stored at −80 °C until the day of use. Final storage buffer was 50 mM HEPES 7, 200 mM NaCl, 0.5 mM EDTA-NaOH 8 and 10% v/v Glycerol.

The BtuF constructs used for substrate transport assays were lacking a 3 C cleavage site, thus the His6-tag remained attached. The protein was purified and desalted into 50 mM Tris 7.5, 200 mM NaCl, 0.5 mM EDTA-NaOH 8 and 10% v/v Glycerol and stored at −80 °C until the day of experiment.

Substrate binding assays with BtuFfluo. The assay is based on monitoring the ligand-induced change in emission of fluorescein attached to BtuF (BtuFfluo). A similar approach has been used to determine the affinity of MBP for maltose in the presence of various sAB60–62. BtuFfluo at 5 mM was incubated with a range of substrate concentrations from 0.02 mM to 33 μM. After incubation for 30 min at 10 °C the fluorescence intensity was recorded at an excitation wavelength of 485 nm and emission at 528 nm. The maximum signal was recorded in the absence of substrate and decreased with increasing substrate concentration. The difference of the maximum intensity minus the signal at a certain substrate concentration was plotted against the substrate concentration. One site–specific binding in Prism GraphPad was used to fit the data for K_d determination (GraphPad Prism version 6.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com”). Binding buffer composition was 50 mM Tris 7.5 and 200 mM NaCl.

References

1. Higgins, C. F. ABC transporters: from microorganisms to man. Annu Rev Cell Biol 8, 67–113 (1992).
2. Swier, L. J. Y. et al. Abc transporters—40 years on, pages cm (Springer, 2016).
3. Rice, A. J., Park, A., & Pinget, H. W. Diversity in ABC transporters: type I, II and III importers. Crit Rev Biochem Mol Biol 49, 426–37 (2014).
4. DeVeaux, L. C. & Kadner, R. J. Transport of vitamin B12 in Escherichia coli: cloning of the btdc region. J Bacteriol 162, 888–96 (1985).
5. Cadieux, N. et al. Identification of the periplasmic cobalamin-binding protein BtuF of Escherichia coli. J Bacteriol 184, 706–17 (2002).
6. Locher, K. P., Lee, A. T. & Rees, D. C. The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. Science 296, 1091–8 (2002).
7. Borths, E. L., Poolman, B., Hvorup, R. N., Locher, K. P. & Rees, D. C. In vitro functional characterization of BtuCD-F, the Escherichia coli ABC transporter for vitamin B12 uptake. Biochemistry 44, 16301–9 (2005).
8. Hvorup, R. N. et al. Asymmetry in the structure of the ABC transporter–binding protein complex BtuCD-BtuF. Science 317, 1387–90 (2007).
9. Korkhov, V. M., Mireku, S. A., Hvorup, R. N. & Locher, K. P. Asymmetric states of vitamin B(12) transporter BtuCD are not discriminated by its cognate substrate binding protein BtuF. FEBS Lett 586, 972–6 (2012).
10. Korkhov, V. M., Mireku, S. A. & Locher, K. P. Structure of AMP-PNP-bound vitamin B12 transporter BtuCD-F. Nature 490, 367–72 (2012).
11. Korkhov, V. M., Mireku, S. A., Veprintsev, D. B. & Locher, K. P. Structure of AMP-PNP-bound BtuCD and mechanism of ATP-powered vitamin B12 transport by BtuCD-F. Nat Struct Mol Biol 21, 1097–9 (2014).
12. Lawrence, J. G. & Roth, J. R. The cobalamin (coenzyme B12) biosynthetic genes of Escherichia coli. J Bacteriol 177, 6371–80 (1995).
13. Di Girolamo, P. M. & Bradbeer, C. Transport of vitamin B12 in Escherichia coli. J Bacteriol 106, 745–50 (1971).
14. Butzin, N. C., Secinaro, M. A., Swithers, K. S., Gogarten, J. P. & Noll, K. M. Thermotoga maritima can salvage cobamidine to synthesize vitamin B12. Appl Environ Microbiol 79, 7006–12 (2013).
15. Borths, E. L., Locher, K. P., Lee, A. T. & Rees, D. C. The structure of Escherichia coli BtuF and binding to its cognate ATP binding cassette transporter. Proc Natl Acad Sci USA 99, 16642–7 (2002).
16. Karpovich, N. K., Huang, H. H., Smith, P. C. & Hunt, J. F. Crystal structures of the BtuF periplasmic-binding protein for vitamin B12 suggest a functionally important reduction in protein mobility upon ligand binding. J Biol Chem 278, 8429–34 (2003).
17. Liu, M., Sun, T., Hu, J., Chen, W. & Wang, C. Study on the mechanism of the BtuF periplasmic-binding protein for vitamin B12. Biophys Chem 135, 19–24 (2008).
18. Fukami-Kobayashi, K., Tateno, Y. & Nishikawa, K. Domain dislocation: a change of core structure in periplasmic binding proteins in their evolutionary history. J Mol Biol 286, 279–90 (1999).
19. Lee, Y. H., Deka, R. K., Norgard, M. V., Radolf, J. D. & Hasemann, C. A. Treponema pallidum TroA is a periplasmic zinc-binding protein with a helical backbone. Nat Struct Biol 6, 628–33 (1999).
20. Berntsson, R. F., Smith, S. H., Schmitt, L., Slotboom, D. J. & Poolman, B. A structural classification of substrate–binding proteins. FEBS Lett 584, 2606–17 (2010).
21. Clarke, T. E., Ku, S. Y., Dougan, D. R., Vogel, H. J. & Tari, L. W. The structure of the ferric siderophore binding protein FhuD complexed with gallichrome. Nat Struct Biol 7, 287–91 (2000).
22. Clarke, T. E., Braun, V., Winkelmann, G., Tari, L. W. & Vogel, H. J. X-ray crystallographic structures of the Escherichia coli periplasmic protein FhuD bound to hydroxamate-type siderophores and the antibiotic albomycin. J Biol Chem 277, 13966–72 (2002).
23. Hu, Y. et al. Crystal structure of a glutamate/aspartate binding protein complexed with a glutamate molecule: structural basis of ligand specificity at atomic resolution. J Mol Biol 382, 99–111 (2008).
24. Wuerger, J., Geremia, S. & Randaccio, L. Structural study on ligand specificity of human vitamin B12 transporters. Biochem J 403, 431–40 (2007).
25. Furger, E., Frei, D. C., Schibli, R., Fischer, E. & Prata, A. E. Structural basis for universal corrinoid recognition by the cobalamin transport protein haptocorrin. J Biol Chem 288, 25466–76 (2013).
26. Kellermann, O. & Szemelcse, S. Active transport of maltose in Escherichia coli K12. Involvement of a “periplasmic” maltose binding protein. Eur J Biochem 47, 139–49 (1974).
27. Miller, D. M., 3rd, Olsen, I. S., Pfleger, J. W. & Quiocio, E. A. Rates of ligand binding to periplasmic proteins involved in bacterial transport and chemotaxis. J Biol Chem 258, 13665–72 (1983).
28. Quiocio, F. A., Spurline, J. C. & Rodseth, L. E. Extensive features of tight oligosaccharide binding revealed in high-resolution structures of the maltodextrin transport/chemosensory receptor. Structure 5, 997–1013 (1997).
29. Wandersman, C., Schwartz, M. & Ferenci, T. Escherichia coli mutants impaired in maltodextrin binding. J Bacteriol 140, 1–13 (1979).
30. Ferenci, T. The recognition of maltodextrins by Escherichia coli. Eur J Biochem 108, 631–6 (1980).
31. Gouridis, G. et al. Conformational dynamics in substrate-binding domains influences transport in the ABC importer GlnPQ. Nat Struct Mol Biol 22, 57–64 (2015).
32. Fulyani, P. et al. Functional diversity of tandem substrate-binding domains in ABC transporters from pathogenic bacteria. Structure 21, 1879–88 (2013).
33. Kräutler, B. Thermodynamic trans-effects of the Nucleotide Base in the B12Coenzymes. Helvetica Chimica Acta 70, 1268–1278 (1987).
34. Geertsma, E. R., Nik Mahmood, N. A., Schuurman-Wolters, G. K. & Poolman, B. Membrane reconstitution of ABC transporters and assays of translocator function. Nat Protoc 3, 256–66 (2008).
35. Wool, J. S., Zeltina, A., Goetz, B. A. & Locher, K. P. X-ray structure of the Yersinia pestis heme transporter HmuUV. Nat Struct Mol Biol 19, 1310–5 (2012).
36. Kabsch, W. Xds. Acta Crystallogr D Biol Crystallogr 66, 125–32 (2010).
37. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr D BiolCrystallogr 66, 213–21 (2010).
38. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. Acta Crystallogr D Biol Crystallogr 66, 486–501 (2010).
39. Winn, M. D. et al. Overview of the CCP4 suite and current developments. Acta Crystallogr D Biol Crystallogr 67, 235–42 (2011).
40. Rizk, S. S. et al. Allosteric control of ligand-binding affinity using engineered conformation-specific effector proteins. Nat Struct Mol Biol 18, 437–42 (2011).
41. de Lorimier, R. M. et al. Construction of a fluorescent biosensor family. Protein Sci 11, 2655–75 (2002).
42. Marvin, J. S. et al. The rational design of allosteric interactions in a monomeric protein and its applications to the construction of biosensors. Proc Natl Acad Sci USA 94, 4366–71 (1997).
43. Karplus, P. A. & Diederichs, K. Linking crystallographic model and data quality. Science 336, 1030–3 (2012).

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Author Contributions
K.P.L. and S.A.M. designed the experiments, analyzed the data and wrote the paper. S.A.M. and T.Z. performed biochemical and structural experiments. V.M.K. was involved in experimental design and fruitful discussions. M.R. and B.K. designed the procedure for mono-cyano-cobinamide synthesis and provided the precursor cob(II)imide. S.A.M. and T.Z. performed biochemical and structural experiments. V. M.K. was involved in experimental design and fruitful discussions. M.R. and B.K. designed the procedure for mono-cyano-cobinamide synthesis and provided the precursor cob(II)imide. S.A.M. and T.Z. performed biochemical and structural experiments.

Additional Information
Accession codes: Structure factors and coordinates are deposited in the Protein Data Bank under the following accession codes: 5M29 (BtuF-Cbi), 5M2Q (BtuF_W66F-Cbi), 5M34 (BtuF_W66Y-Cbi) and 5M3B (BtuF_W66L-Cbi).

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