Doctoral Thesis

Structural and Functional Studies of the Vitamin B12 Uptake System BtuCD-F in Escherichia coli

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Structural and Functional Studies of the Vitamin B12 Uptake System BtuCD-F in *Escherichia coli*

A thesis submitted to attain the degree of

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(Dr. sc. ETH Zurich)

presented by

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2017
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# Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AMPPNP</td>
<td>Adenosine-5'-(β,γ)-imido]triphosphate</td>
</tr>
<tr>
<td>ARS</td>
<td>ATP-regenerating system</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Analytical ultracentrifugation</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cbi</td>
<td>Cobinamide</td>
</tr>
<tr>
<td>Cbl</td>
<td>Cyanocobalamin</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CV</td>
<td>column volume</td>
</tr>
<tr>
<td>DDM</td>
<td>Dodecyl-β-D-maltopyranoside</td>
</tr>
<tr>
<td>DMB</td>
<td>5,6-dimethylbenzimidazole</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECF</td>
<td>Energy-coupling factor</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen-binding</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>His-tag</td>
<td>Histidine affinity tag</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized Metal Affinity Chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LDAO</td>
<td>N,N-dimethyldodecylamine N-oxide</td>
</tr>
<tr>
<td>LLO</td>
<td>lipid-linked oligosaccharide</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose-binding protein</td>
</tr>
<tr>
<td>MST</td>
<td>Microscale thermophoresis</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide-binding domain</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-nitritriaceticacid</td>
</tr>
<tr>
<td>OD₆₀₀</td>
<td>Optical density at 600 nm</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SBP</td>
<td>Substrate-binding protein</td>
</tr>
<tr>
<td>SBD</td>
<td>Substrate-binding domain</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific broth</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco Etch Virus</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
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</table>
Summary

Membrane transport is a crucial process for living cells. Active transport against a concentration gradient requires energy gained from ATP hydrolysis. ATP-binding cassette (ABC) transporters are a super-family of integral membrane proteins that couple the energy of ATP binding and hydrolysis to substrate translocation. ABC importers are a sub-family responsible for the uptake of essential nutrients and consist of a membrane-bound transporter and a substrate-binding protein (SBP). The ABC importer BtuCD-F catalyzes vitamin B12 transport into the cytoplasm of E. coli and extensive structural and functional studies have resulted in a detailed description of its transport mechanism. Nevertheless, the molecular basis of the interface between the SBP BtuF and the transporter BtuCD as well as the interaction of the substrate with the individual transporter components are poorly understood. Early in vivo data suggested that the vitamin B12 uptake system in E. coli also transports cobinamide (Cbi), a compound related to B12 but considerably smaller.

In this thesis, I demonstrate BtuCD-F-catalyzed in vitro Cbi transport and compare it to the one of B12. This required radiolabeling of Cbi and establishing of a liposome-based transport assay for accurate quantification. A mutagenesis study of residues at strategic positions within the translocation pathway revealed the distinct interaction with the two substrates. The development of an E. coli spheroplast-based transport assay will allow efficient screening of BtuCD mutants in the future. A new fluorescence-based binding assay was developed to compare Cbi and B12 binding to BtuF and the crystal structure of Cbi-bound BtuF revealed a key residue facilitating binding of the smaller substrate Cbi. High-resolution structures of distinct states of the full BtuCD-F complex ideally bound to B12 or Cbi would reveal the molecular details of the interactions. I explored two strategies, including co-crystallization with a Fab fragment and engineered disulfide cross-linking of BtuF to BtuCD. The results, however, indicated that these samples were not optimal for structural studies using X-ray crystallography, but can be potential targets for cryo-electron microscopy.

In a final approach, I studied nanobody-mediated inhibition of ABC importers using BtuCD-F as a model system. I generated BtuF-specific nanobodies and identified an inhibitory binder using the developed biochemical assays. The crystal structure of the nanobody-BtuF complex revealed the molecular basis of the observed inhibitory effects and serves as a promising starting point for affinity maturation. This study provided the framework for nanobody-mediated inhibition of an ABC importer and application to the import systems of pathogenic bacteria might result in novel antibiotic strategies.
Zusammenfassung


1. Introduction

Cellular transport across biological membranes is a prerequisite of life. Living cells have developed distinct mechanisms to take up essential nutrients and extrude metabolic waste or toxic compounds. Transport of a compound along an electrochemical gradient is energetically favorable and defined as downhill or passive transport. Small non-polar molecules like oxygen, carbon dioxide, nitrogen or water are free to diffuse across biological membranes, which in case of water is the basis for osmosis. Integral membrane proteins can be involved in the diffusion of specific molecules and thus facilitate their transport by acting as channels. Examples of channels are the aquaporins facilitating water diffusion or GLUT4 mediating diffusion driven glucose uptake [1-3]. Due to the charged nature of ions, passage through the hydrophobic membrane requires specific channels, facilitating ion movement along their electrochemical gradient [4]. Such ion channels are exemplified by the ligand-gated ion channels crucial for signal transmission in the nervous system [5].

Transport against the electrochemical gradient of the substrate requires energy and is described as uphill or active transport. The energy is either gained by ATP hydrolysis (primary active transport) or by coupling the energetically un-favored transport to a second energetically favored transport (secondary active transport). Secondary active transporters can be classified based on the direction of transport of their two substrates: symporters (same direction) and antiporters (opposite direction) [6]. Membrane protein families facilitating primary active transport are P-, F- and V-type ATPases and ATP-binding cassette (ABC) transporters [7]. In this work the focus lies on a member of the ABC transporter family mediating vitamin B12 uptake in Escherichia coli.

1.1 ATP-binding cassette (ABC) transporter family

1.1.1 Introduction to ABC transporters

ABC transporters are a big family of integral membrane proteins represented in all kingdoms of life [8-11]. They are involved in nutrient uptake, drug extrusion, and lipid homeostasis and couple the free energy of ATP binding and hydrolysis to substrate translocation across a lipid bilayer. They are classified as importers or exporters depending on the direction of transport into or out of the cytoplasm. Importers have only been described in prokaryotes and plants so
far, but exporters are found in various prokaryotes and eukaryotes [12, 13]. Multi-drug resistance associated with ABC exporters has been described in bacterial and eukaryotic cells [14]. The human ABC exporter P-glycoprotein (P-gp or ABCB1) is responsible for the extrusion of hydrophobic substrates and causes multidrug resistance in cancer therapy [15]. Similar multidrug resistance has been observed for ABCG2, another constitutively expressed human ABC exporter. Several human genetic diseases including cystic fibrosis, Stargardt disease, age-related macular degeneration or adrenoleukodystrophy are associated with the mal-functioning of ABC exporters, reflecting the pharmacological relevance of this protein family [16]. The most common cause of cystic fibrosis is a single amino acid deletion in the cystic fibrosis transmembrane conductance regulator (CFTR), which is involved in the regulation of mucus on epithelial cells [17]. Despite the diverse functions of ABC transporters, they share a common basic architecture.

1.1.2 ABC transporter architecture

ABC transporters are composed of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) located in the cytoplasm (Fig. 1.1A). The NBDs are conserved and provide the energy to power substrate transport by binding and hydrolyzing ATP. The NBD dimer can adopt two conformations, an open conformation in the nucleotide-free state and a closed one in the nucleotide-bound state [18]. An additional translational shift along the dimer interface occurs when switching from the open to the closed state [19]. The ATP binding site is located at the interface of the two NBD subunits, which are arranged in a head-to-tail orientation. Most ABC transporters have two canonical ATP binding sites, but some exporters have only one functional site. Each NBD contains two sub-domains, an α-helical sub-domain and a Rec-A like sub-domain. Conserved features of the NBDs are the Walker A (or P loop), the Walker B, the Q, D, and H loops, and the ABC signature motif LSGGQ (Fig. 1.1B, [20, 21]). ATP binds between the Walker A of one NBD and the LSGGQ motif of the other NBD. A catalytically essential glutamate in the Walker B functions as the catalytic base and increases the nucleophilicity of the attacking water to cleave off the ATP γ-phosphate. A bound Mg(II) ions is responsible for proper positioning of the phosphate groups and the histidine in the H-loop (switch histidine) stabilizes the transition-state geometry. Nucleotide-induced closing of the NBD dimer brings the D loops (dimerization loops) together.
Figure 1.1: Architecture of ABC transporters. A) The basic domain arrangement found in ABC exporters and importers. Two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) assemble to form the full transporter. The direction of substrate translocation is indicated with a red arrow. The coupling helices mediating the conformational changes between the NBDs and the TMDs are labeled. B) Illustrated is the nucleotide-binding domain (NBD) with the conserved features. See main text for the description of the conserved features. Figure from [20].

In the TMD, the only structurally conserved feature is the coupling helix, which mediates the conformational changes between the NBD (via Q loop) and TMD [22]. The TMDs contain the substrate translocation pathway and are very diverse due to the broad substrate range of ABC transporters. The number of transmembrane helices and the topology differ between exporters and importers as well as the proposed transport mechanisms [20].

1.1.3 ABC exporters

ABC exporters are found in all kingdoms of life. 48 human ABC exporters have been described and divided into seven subfamilies (A-G) [23]. Exporters can be formed by one polypeptide chain consisting of two TMDs and two NBDs, or alternatively, by the assembly
of two polypeptide chains, each consisting of one TMD and one NBD. The structure of the *Staphylococcus aureus* multidrug exporter Sav1866 represents the architecture also found in the B-subfamily of human ABC exporters (Fig. 1.2A, [24, 25]). The recently determined structure of the human sterol transporter ABCG5/ABCG8 [26], revealed a distinct fold of the G-subfamily (fig. 1.2B). Both families contain twelve transmembrane helices in the assembled transporter, but the topology of the TMDs and the distance between the TMDs and NBDs are different. The fold of the B-subfamily is represented by elongated TMDs and crossing-over of the helices from the two TMDs has been observed. From the middle of the TMDs, the helices diverge into two wings, each consisting of TM1-2 from one subunit and TM3-6 from the other subunit. In the G-subfamily, no crossing-over of the helices of the two TMDs has been described. Within each half-transporter, a horizontal cytoplasmic helix right below the membrane was defined as the connecting helix, linking the NBD to the TMD. Structures of the B-subfamily in distinct states have led to the formulation of two transport mechanisms (Fig. 1.3).

**Figure 1.2: Structural representation of the two distinct folds observed in ABC exporters.** A) Shown is the fold of B-subfamily exporters using *S. aureus* Sav1866 as a representative (PDB ID 2HYD). B) Illustrated is the fold of G-subfamily exporters represented by the human sterol transporter ABCG5/ABCG8 (PDB ID 5DO7). Cartoon representation was used and the half-transporters are colored in blue and green.
In an alternating access mechanism described for multi-drug exporters, the substrate binds to the inward-facing state 1 of the transporter either from the cytoplasm or from the membrane (Fig. 1.3A). ATP binding triggers the closing of the NBDs and the substrate translocation pathway resets into an occluded state 2, which is closed to both sides of the membrane, before adopting an outward-facing state 3 for substrate release. ATP hydrolysis induces conformational changes in the NBDs, which are converted to the TMDs via the coupling helices, resetting the transporter to the inward-facing state 1. It has been hypothesized that the inward-facing state contains a substrate-binding site, which is absent in the outward-facing state facilitating substrate release.

In an outward-only mechanism proposed for the lipid-linked oligosaccharide flippase PglK [20, 27], the transport reaction starts with bound ATP in the closed NBD dimer and an outward-facing conformation of the translocation pathway represented by state 1 (Fig. 1.3B). While the lipid tail of the substrate remains associated with the transporter at the interface to the membrane, the pyrophosphate-glycan headgroup binds to an arginine patch in the TMDs, forming strong electrostatic interactions shown in state 2. ATP hydrolysis and inorganic phosphate release leads to a medium opening of the NBD dimer and closing of the external wings of the TMDs, which results in substrate extrusion as illustrated by state 3. Exchange of ADP with ATP and binding of a new substrate resets the transporter to state 1.

Figure 1.3: Two distinct transport mechanisms of ABC exporters with the B-subfamily fold. Shown are an alternating access mechanism proposed for multi drug extrusion systems (A) and an outward-only mechanism suggested for lipid-linked oligosaccharide flippases (B). See main text for description. Figure from [20].
1.2 ABC importers

1.2.1 Substrate-binding proteins

Prokaryotic importers are responsible for the uptake of essential nutrients into the cytoplasm. They depend on substrate-binding proteins (SBPs) for delivery of the cargo to the membrane-embedded transporters [28]. The SBP can be a soluble protein located in the periplasm in case of Gram-negative bacteria or tethered to the membrane via a lipid or protein anchor observed in Gram-positives or archaea. Alternatively, additional domains fused to the TMDs, called substrate-binding domains (SBDs), can serve as the substrate-binding partners [29, 30]. SBPs were initially divided into three classes and extended to six major clusters based on their structural similarities (Fig. 1.4, [31, 32]). In the past six years, the amount of unique SBP structures increased 5-fold, leading to the recent addition of a cluster [33]. The basic architecture of SBPs are a N- and a C-terminal domain (N- and C-lobe), which are connected by an interdomain helix or loops. The two domains are related by a pseudo-twofold symmetry and consist of a certain set of α-helices and β-sheets. A venus-fly trap or induced fit binding mechanism has been postulated, in which the SBP is open in the ligand-free state and closed in the ligand-bound state. Whether ligand binding induces closure or the closed state binds the ligand is unclear. However, all SBPs are the main determinant for substrate specificity of the corresponding import system. Based on their TMD topology, importers have been classified into type I, type II and ECF-type (energy-coupling factor) importers with distinct transport mechanisms [20, 22, 28, 34].

Figure 1.4: Structural classification of substrate binding proteins. Shown are the six main clusters of SBPs. The helix or loops connecting the N- and C-terminal domains are shown in orange. Figure from [31].
1.2.2 Type I importers

Type I importers are responsible for the uptake of sugars, amino acids or transition metals and bind their substrate with micromolar affinity. The best-studied member is the *E. coli* maltose/maltodextrin transporter MalFGK$_2$-MBP (Fig. 1.5, [35-41]). MBP, or MalE, is the maltose binding protein and located in the periplasm. Two individual proteins, MalF and MalG, form the TMDs, and two MalK subunits form the NBDs. The TMDs and NBDs are non-covalently associated. Type I importers have a set of ten core TM helices in the assembled dimer, five per subunit, but larger numbers have been observed for MalFGK$_2$ or ModBC from *Archaeoglobus fulgidus* [42]. MalFGK$_2$-MBP has been crystallized in different states of the transport cycle, which led to the formulation of a detailed transport mechanism (Fig. 1.6). Maltose-loaded MBP binds to the transporter in the inward-facing state 1, which leads to the transition into a pre-translocation state 2 with semi-closed NBDs. ATP-binding induces conformational changes in the NBDs and TMDs, resulting in an outward-facing state 3 with a continuous translocation pathway between MBP and the TMDs. Since the TMDs have a defined substrate-binding site, unidirectional transport is ensured. ATP hydrolysis and release of the products resets the transporter into the inward-facing state 1 and the substrate is translocated into the cytoplasm. Additional regulatory domains have been described for type I importers, which bind the transported substrate and thus regulate the transport activity by a mechanism termed trans-inhibition [43-45].

**Figure 1.5: Structural representation of a type I importer.** Crystal structure of MalFGK$_2$-MBP from *E. coli* (bound to ATP and maltose; PDB ID 2R6G). MBP is shown in brown, MalF and MalG in green and blue, and the two MalK subunits in pink and yellow.
leads to futile ATP hydrolysis (state 5). Adapted from [35].

1.2.3 ECF-type importers

ECF-type importers are responsible for the uptake of vitamins [46]. The crystal structure of a folate ECF transporter from *Lactobacillus brevis* revealed a distinct composition of these ABC importers [47]. The substrate-binding component (S-component) of ECF-type importers is unlike the SBP of the other importers types an integral membrane protein [48, 49]. The assembled transporter is formed by the association of the S-component with its ECF module, which consists of a transmembrane part and two NBDs, EcfA and EcfA’ (Fig. 1.7A). The ECF part can interact with one S-component (class I) or with several S-components with distinct substrate specificity (class II). ECF-type importers have only been described recently, but based on the crystal structures of two distinct states a transport mechanism has been proposed (Fig. 1.7B). The S-component binds its substrate on the outside, closes its substrate-binding site and reorients in the membrane (actions 3 and 5). ATP hydrolysis and product release prepares the ECF module for binding to the substrate-loaded S-component (action 4). Assembly of the full transporter leads to substrate release into the cytoplasm (action 6) and ATP binding results in the dissociation of the assembled transporter (action 1 and 2).
Figure 1.7: Structural representation and transport mechanism of ECF-type importers. A) Crystal structure of the folate ECF transporter from *Lactobacillus brevis* (PDB ID 4HUQ). The S-component is shown in blue and the ECF module with a green membrane part and pink and yellow NBDs. B) Toppling mechanism of ECF-type importers. See main text for description. Figure from [50].

1.2.4 Type II importers

Type II importers are responsible for the uptake of transition metals and micronutrients. The SBP binds the metal directly or chelated in siderophores, heme or vitamin B12. Unlike type I importers, the SBP of type II importers binds the substrate with nanomolar affinity [34]. The structures of three distinct members have been solved and showed that one TMD is composed of ten TM helices, which sums up to a total of twenty TM helices in the assembled transporter [51-53]. For the vitamin B12 transporter BtuCD, two BtuC subunits form the TMDs and two non-covalently associated BtuD subunits build the NBDs (Fig. 1.8). BtuF is the soluble periplasmic SBP and belongs to cluster A of the SBPs [54-56]. The first type II structures were inconsistent and made it difficult to formulate a general transport mechanism, because distinct conformations were observed in the TMDs. The nucleotide-free state of *E. coli* BtuCD showed an outward-facing conformation of the TMDs and the nucleotide-free state of *Haemophilus influenzae* HI1470/71 was inward-facing. The structure of *Yersinia pestis*
HmuUV confirmed the outward-facing state in the absence of nucleotide [53]. The crystal structure of BtuCD in complex with the SBP BtuF revealed an asymmetric conformation of the translocation pathway with one outward-facing and one inward-facing TMD. A detailed transport mechanism for type II importers could only be formulated after the nucleotide-bound states of BtuCD (with and without BtuF) were visualized and accompanied by extensive functional studies (discussed in chapter 1.3).

**Figure 1.8: Structural representation of BtuCD and BtuCD-F.** 
A) Illustrated is nucleotide-free BtuCD in the outward-facing state (PDB ID 1L7V). The two BtuC subunits are colored in green and blue and the two BtuD subunits in yellow and pink. B) Nucleotide-free BtuCD in complex with BtuF, which is colored in brown (PDB ID 2QI9). The green BtuC subunit adopts an inward-facing conformation and the blue BtuC subunit is outward-facing. A periplasmic gate and cytoplasmic gate I are indicated.
1.3 **The vitamin B12 transporter BtuCD-F in *E. coli***

1.3.1 **B12 uptake in *E. coli***

Vitamin B12 or cyanocobalamin (Cbl) is the most complex of all vitamins. Many organisms cannot synthesize it de novo and depend on external supply from the environment. In the Gram-negative bacteria *E. coli*, B12 transport across the outer membrane is facilitated by the β-barrel membrane protein BtuB [57]. The transport is powered by the TonB-ExbB-ExbD complex using the proton gradient as energy source (Fig. 1.9). Once in the periplasm, B12 is bound by BtuF with nanomolar affinity and delivered to BtuCD located in the inner membrane [51, 58-60]. Transport across the inner membrane is powered by ATP hydrolysis.

![Vitamin B12 transport in E. coli](image)

**Figure 1.9: Vitamin B12 transport in *E. coli***. Assimilation of B12 from the environment is a two-step process of active transport. The TonB-ExbB-ExbD-dependent B12 transport across the outer membrane is facilitated by BtuB. B12 transport across the inner membrane is catalyzed by BtuCD-F using ATP hydrolysis as energy source. Figure from [60].
1.3.2 Substrate transport mechanism for type II importers

Based on the amount of structural and functional data available for BtuCD-F, a detailed B12 transport mechanism has been formulated [51, 55, 61-66]. The structures of BtuCD-F in distinct states of the transport cycle allowed the identification of a periplasmic gate and a cytoplasmic gate I and II (Fig. 1.8 and 1.10). The periplasmic gate is located at the interface to BtuF and seals the translocation pathway from the periplasm. The cytoplasmic gates are responsible for the opening and closing of the translocation pathway towards the cytoplasm. Conformational changes in these gates are crucial for the BtuCD-F-catalyzed transport mechanism.

The proposed mechanism describes the coupling of nucleotide binding and hydrolysis and BtuF-induced conformational changes necessary for translocation of the substrate [63, 64]. Due to the high cytoplasmic ATP concentration, BtuCD is generally bound to nucleotide, which results in an outward-facing state 1 with a closed cytoplasmic gate II and an open cytoplasmic gate I (Fig. 1.10). Binding of B12-loaded BtuF results in the transfer of B12 into a substrate cavity within the TMDs. In this state 2, the cavity is closed to both sides of the membrane by the periplasmic gate and the cytoplasmic gate II. The sealed cavity is a prerequisite to ensure unidirectional transport, since no high-affinity binding site is present within the translocation pathway. ATP hydrolysis and product release leads to the opening of cytoplasmic gate II and translocation of B12 in a peristalsis-like mode, resulting in an inward-facing state 3. The transiently populated state 3 adopts an asymmetric state 4, in which one BtuC subunit is outward-facing and one is inward-facing, ensure no leaking of other components. Binding of ATP releases BtuF and resets BtuCD into the outward-facing state 1. Alternatively, spontaneous unbinding of BtuF results in an outward-facing BtuCD with closed cytoplasmic gate I and binding of ATP to this state 5 resets the transporter into state 1. The crystal structure of each state was solved for BtuCD-F except for state 3. This conformation was recently visualized for the Burkholderia cenocepacia heme transporter BhuUV-T, thus providing the structural evidence [67].
Figure 1.10: BtuCD-F catalyzed B12 transport mechanism. The transporter is displayed in cylinder representation with BtuC, BtuD and BtuF shown in grey. The gating helices are depicted in blue and form the periplasmic gate and cytoplasmic gate I. Magenta brackets illustrate cytoplasmic gate II. ATP colored in yellow and B12 in red are shown in ball-and-stick model. Figure from [64].

Deciphering the B12 transport mechanism

Structure determination of the nucleotide-bound states required stabilization of BtuCD using two mutations in BtuD. A hydrolysis-deficient substitution E159Q was necessary and co-crystallization was performed with Adenosine-5’-[(β,γ)-imido]triphosphate (AMPPNP), a non-hydrolyzable ATP analog. The second mutation, N162C, was an engineered disulfide bond between the two BtuD subunits to stabilize the NBD dimer formation. In my previous work, I performed the functional characterization of wild-type BtuCD-F and the mutants using ATP hydrolysis and B12 transport assays. The results of these experiments were published together with the structure of AMPPNP-bound BtuCD-F in Nature 2012 [63]. To
investigate the conformational changes induced by BtuF binding to AMPPNP-bound BtuCD, the structure of the transporter was also solved in the absence of BtuF. The functional effect of nucleotide on BtuF binding was investigated with binding assays for which I designed and prepared a fluorescently labeled BtuF construct (BtuF_{flu}). I was also involved in BtuCD sample preparation in detergent or in nanodiscs used for the binding assays, and contributed to the binding assays using microscale thermophoresis (MST) and analytical ultracentrifugation (AUC). The results of these experiments were published together with the AMPPNP-bound BtuCD structure in Nature Structural and Molecular Biology 2014 [64].

### 1.4 Open questions and aim of the thesis

The structures of BtuCD-F in distinct states of the transport cycle allowed the formulation of the presented transport mechanism. The coupling of nucleotide binding and hydrolysis and the BtuF induced conformational changes in the transporter were described. However, BtuF and the molecular details of the BtuF-BtuC interface were poorly resolved in the electron density of the different structures. The lack of BtuF mediated crystal contacts and the flexible binding of BtuF to BtuCD were potential reasons. To address this issue, I explored two strategies to obtain higher resolution of distinct states of BtuCD-F. The first strategy was co-crystallization with a BtuF-binding Fab fragment and the second strategy was engineered disulfide cross-linking of BtuF to BtuCD.

Before the individual *btuC*, *btuD* and *btuF* genes were identified, *in vivo* transport data suggested that the vitamin B12 transport system in *E. coli* could also transport cobinamide (Cbi) [68]. B12 and Cbi are related but differ in their size and chemical properties. *In vitro* Cbi transport by BtuCD-F was not demonstrated and the interaction of the translocated substrates with the individual components of the transport system was poorly understood. The B12-bound BtuF structure revealed the molecular organization of the substrate-binding site, but how BtuF would bind the smaller substrate Cbi was unknown. If the BtuC translocation pathway senses the substrate and thus contributes to substrate specificity was also not known.

In a third approach, I used BtuCD-F as a model system to explore its inhibition by nanobodies. Inhibition of an ABC type II importer by a Fab fragment was recently described and I aimed to explore the feasibility of nanobody-mediated inhibition [69].
Outline of the thesis

In chapter 2, I describe the development of quantitative assays to investigate in vitro substrate binding and transport. In vitro transport assays required radioactive substrate and reconstitution of BtuCD into liposomes including its characterization. A method was developed to synthesize radiolabeled Cbi, because it was not commercially available. Crystallization of Cbi-bound BtuF revealed the molecular basis of the Cbi-BtuF interaction. A fluorescence-based substrate-binding assay was used to analyze a key residue observed in the crystal structure.

In chapter 3, the established in vitro transport assay was used to compare Cbi and B12 transport. A mutagenesis study was performed to probe the function of semi-conserved residues at strategic positions within the translocation pathway. The functional integrity of the mutants were analyzed by ATP hydrolysis and BtuF binding assays. Additionally, an E. coli spheroplast-based transport assay was established to screen the effect of BtuCD mutants in an high-throughput manner.

In chapter 4, the feasibility of nanobody-mediated inhibition of BtuCD-F was explored. BtuF-specific nanobodies were generated and investigated for their effects on substrate binding and transport, using the binding assay developed in chapter 2 and the spheroplast-based transport assay in chapter 3.

In chapter 5, concluding remarks and an outlook are presented.

The appendix describes two tools to obtain higher resolution structures of BtuCD-F in distinct states. The two strategies were engineered disulfide cross-linking of BtuF to BtuCD and targeting of BtuF with a Fab fragment.

References


2. Structural and functional investigation of BtuCD-F as transporter of cobinamide

This study has been published in “Conformational Change of a Tryptophan Residue in BtuF Facilitates Binding and Transport of Cobinamide by the Vitamin B12 Transporter BtuCD-F.” by Mireku SA, Ruetz M, Zhou T, Korkhov VM, Kräutler B, Locher KP. Sci Rep. 2017

Author contributions are indicated in section 2.3 Experimental Procedures.

2.1 Abstract

BtuCD-F 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.

2.2 Introduction

E. coli is unable to synthesize cobalamin de novo. However, it expresses enzymes that facilitate its synthesis from the precursor cobinamide (Cbi) [1], which lacks the 5,6-dimethylbenzimidazole (DMB) moiety and phosphoribosyl linker and is therefore smaller (Fig. 2.1). Before the individual proteins (BtuC, BtuD, BtuF) were identified, in vivo transport data suggested that the E. coli uptake system specific for cyanocobalamin (Cbl) also transports Cbi [2]. In line with this observation, recent in vitro binding data showed that
Thermotoga species BtuF variants can bind not only Cbl, but also Cbi [3]. 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 [4, 5], it is not known whether and how E. coli BtuF would recognize and bind Cbi in addition to Cbl. Also, Cbi transport by BtuCD-F was never demonstrated.

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. Our results showed that Cbi is a bona fide substrate of the BtuCD-F transporter.

Figure 2.1: Chemical structures and cobalamin synthesis from cobinamide. Shown are the chemical structures of Cbl (A) and Cbi (B). Cobalamin synthesis requires the attachment of DMB and a phosphoribosyl to the precursor Cbi. Note that the cyanide group of mono-cyano Cbi can be on the α- or β-side of the corrin ring (R = CN or H₂0).

C) Illustrated is the cobalamin biosynthetic pathway. E. coli genes encoding the required enzymes are indicated. Cbl, cyanocobalamin; Cbi, cobinamide; DMB, 5,6-dimethylbenzimidazole; Ado, adenosyl; CN, cyano; FMN, flavin mononucleotide; UroIII, uroporphyrinogen III. Figure

adapted from [1].
2.3 Experimental Procedures

2.3.1 Generation of radiolabeled cobinamide

(Markus Ruetz and Bernhard Kräutler established the method for radiolabeling and produced the precursor cob(II)inamide)

Cob(II)inamide was synthesized using a previously described method [6] and $^{14}$C-cyanide-labeled through an oxidative reaction with K$^{14}$CN (Perkin Elmer NEC079H001MC, 1 mCi, 58.6 mCi/mmol) generating $^{14}$C-cyano-aquo-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 K$^{14}$CN was added in multiple steps to an aqueous solution containing 4.8 µmol aquo-cob(II)inamide at 22°C. The conversion of cob(II)inamide into $^{14}$C-cyano-aquo-cobinamide was followed by recording a 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 K$^{14}$CN stock dilutions as calibration curve. Products in the 5:5 MeOH:H$_2$O eluent were used for transport assays and stored at -20°C.

2.3.2 BtuCD expression and purification

A cys-less wild-type BtuCD expression construct (no solvent accessible cysteine residues, Fig. 2.2) was generated by M. Niederer and R. Hvorup and published in [7]. BtuCD was expressed as described previously in [8]. In brief, expression was done in E. coli BL21-Gold (DE3) cells cultured in Terrific broth (TB) medium supplemented with 0.1 mg/ml ampicillin and 1 % (w/v) glucose. The culture was grown in 5 l baffled flasks at 37 °C until it reached an OD$_{600}$ of 3. Protein expression was induced with 0.25 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 1.5 hours. Cells were harvested by centrifugation (8k rpm, SLC-6000 rotor, 10 min, 4 °C) and pellets were stored at -80 °C.
Figure 2.2: BtuCD expression construct (Bh). The plasmid (pKL7A) contained cys-less btuD and btuC in tandem for the expression from one mRNA. The btuC gene was fused to an N-terminal His$_{10}$-tag with a TEV protease cleavage site. Restriction sites are indicated. T7, T7 promotor; lacO, lac operator; RBS, ribosomal binding site.

BtuCD was purified as described previously with some modifications regarding buffer composition [8, 9]. The frozen cell pellet (5 – 10 g) was resuspended at a ratio of 1:10 cell mass to buffer volume using a homogenizer at 3.6k rpm (IKA® T25 digital ULTRA-TURRAX®). The solubilization buffer contained 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 25 mM imidazole-HCl pH 8 and 1 % (w/v) N,N-dimethyldodecylamine N-oxide (LDAO, Anagrade). The cells were disrupted on ice by sonication (3 × 2 min, 50 % duty cycle, output control 6) and BtuCD was solubilized from the membrane by stirring for 1.5 hours at 4 °C. The sample was cleared by centrifugation (16k rpm, SA-600 rotor, 30 min, 4 °C) and BtuCD was immobilized on a Ni-NTA resin equilibrated with 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 25 mM imidazole-HCl pH 8 and 0.1 % (w/v) LDAO. The column was washed with the same buffer followed by a second washing step with 50 mM imidazole-HCl pH 8. The protein was eluted with 200 mM imidazole-HCl pH 8 and the buffer was exchanged to 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 0.1 % (w/v) LDAO using a HiPrep 26/10 desalting column (GE Healthcare). The protein sample and buffers were always kept on ice or at 4 °C.

2.3.3 BtuCD reconstitution into liposomes

BtuCD was reconstituted as published in [8] and based on a general protocol for membrane reconstitution of ABC transporters [10]. Purified protein was used directly for reconstitution into liposomes consisting of E. coli Polar lipids and α-Phosphatidylcholine (3:1 weight ratio, Avanti Polar Lipids 100600C and 840051C). Liposomes were destabilized with 0.3 % (w/v)
Chapter 2

Triton X-100 for 20 min at RT. Protein was added with a 1:50 weight ratio (protein to lipid) and incubated for 90 min at RT. Detergent was removed by BioBeads (40 mg/ml mixture) in four steps. The proteoliposomes were resuspended in 10 mM Tris-HCl pH 7.5, 100 mM NaCl and stored at -80°C. The protein concentration in the liposomes was determined by Amido black protein assay using BSA for the calibration curve.

2.3.4 Orientation of BtuCD in liposomes

The amount of right-side-in oriented BtuCD in liposomes (BtuD in lumen) was determined as described previously for ABC type II importer HmuUV with minor modifications [11]. Briefly, liposome-reconstituted BtuCD was incubated with 1 µM fluorescently labeled BtuF (BtuF_fluo) for 30 min at RT followed by a spin (55k rpm, TLA-55 rotor, 20 min, 4 °C) to remove excess BtuF_fluo. The pellet was washed with 10 mM Tris-HCl pH 7.5, 100 mM NaCl and resuspended at 1.25 mg/ml liposome concentration. The amount of BtuF_fluo associated with the liposomes was determined by measuring the fluorescence signal at an excitation wavelength of 488 nm and an emission wavelength of 516 nm. Unspecific interaction of BtuF_fluo was measured using empty liposomes. Two alternative methods to determine the orientation of transporters in liposomes can be used. They are based on determining the amount of NBDs available for proteolytic digest or labeling of an engineered cysteine with a thiol-specific reagent [12].

2.3.5 Substrate transport assay with liposomes containing BtuCD

To prepare the proteoliposomes for the transport assays, 2 mM ATP (Sigma) and an ATP-regenerating system (ARS) were incorporated into the lumen by three freeze-thaw cycles. The purpose of the ARS was to keep a constant ATP concentration within the time course of the experiment. It contained 2.4 mg/ml creatine-kinase (Roche), 24 mM phosphocreatine (Roche), 5 mM MgCl₂, 20 mM Tris-HCl pH 7.5 and 150 mM NaCl. Unilamellar proteoliposomes of defined size were generated by extrusion (11 times) through a 400 nm polycarbonate membrane (Avanti Polat Lipids). Proteoliposomes were pelleted by ultracentrifugation (50k rpm, TLA-55 rotor, 20 min, 4°C), then washed and resuspended in outside buffer containing 20 mM Tris-HCl pH 7.5, 200 mM NaCl and 5 mM MgCl₂. The proteoliposomes ready for the transport assay were kept on ice to minimize ATP hydrolysis.
until the reaction start. For the substrate transport assays radiolabeled $^{14}$C-cyano-aquo-cobinamide ($^{14}$CN-Cbi, labeled in house) or $^{57}$Co-cyanocobalamin ($^{57}$Co-Cbl, MP Biomedicals 06B-430000, 10.5 uCi) were used. Prior to reaction start substrate-loaded BtuF and BtuCD containing liposomes were incubated separately for 30 min to equilibrate to RT. The transport reaction was started by the addition of substrate-loaded BtuF to the BtuCD-containing liposomes with final concentrations of 15 µM substrate, 1 µM BtuF and 4 mg/ml liposomes (~ 0.5 µM total BtuCD). The transport reaction was stopped by diluting 50 µl samples in 300 µl ice cold stop buffer composed of outside buffer with 8 % (w/v) PEG6000 and 100 µM unlabeled Cbl. Samples were transferred to a manifold filtration system (Millipore MSFBN6B) and washed twice with 200 µl cold stop buffer. The radioactivity trapped on the filters was measured either with a β counter using liquid scintillation counting for $^{14}$CN-Cbi or a γ counter for $^{57}$Co-Cbl. Initial transport rates were determined by using linear regression in Graphpad Prism 7.

### 2.3.6 BtuF cloning, expression and purification

The basic BtuF construct was cloned by R. Hvorup and modified to a C-terminal His$_6$-tag version by V. Korkhov (Fig. 2.3, [13]). The construct was converted to a 3C cleavable His$_6$-tag by introducing the cleavage sequence at the BamHI site using oligo duplex cloning. All discussed point mutations in this chapter were introduced by site-directed mutagenesis (Invitrogen QuickChange). Every modification to the construct was confirmed by DNA sequencing (Microsynth AG).

**Figure 2.3: BtuF expression construct.** The *btuF* gene (in pKL4) was preceeded by an OmpA secretion signal for periplasmic secretion and fused to a C-terminal His$_6$-tag.
BtuF was expressed in *E. coli* BL21-Gold (DE3) cells grown in 5 l baffled flasks containing TB medium supplemented with 0.1 mg/ml ampicillin and 1 % (w/v) glucose [7, 13]. Cultures were incubated at 37 °C until they reached an OD$_{600}$ of about 0.8. The temperature was reduced to 25 °C and 30 min later the expression was induced with 0.25 mM IPTG. Cells were harvested after 4 hours by centrifugation (8k rpm, SLC-6000 rotor, 10 min, 4 °C) and the pellet was stored at -80 °C.

BtuF was purified as described previously [8, 13]. The frozen cell pellet was resuspended at 1:10 cell mass to buffer volume in 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 25 mM imidazole-HCl pH 8 and 10 % (v/v) glycerol. A rod homogenizer was used for resuspension at 3.6k rpm (IKA® T25 digital ULTRA-TURRAX®). The homogenate was sonicated on ice (3×2 min, 50 % duty cycles, output control 6) and the cell debris was removed by centrifugation (16k rpm, SA-600 rotor, 30 min, 4°C). BtuF was immobilized on a Ni-NTA slurry equilibrated with 50 mM Tris-HCl pH 7.5, 200 mM NaCl and 25 mM imidazole-HCl pH 8. The column was washed with the same buffer followed by a second wash with 50 mM imidazole-HCl pH 8. BtuF was eluted with 200 mM imidazole-HCl pH 8 and the buffer was exchanged to 50 mM Tris 7.5, 200 mm NaCl, 0.5 mM EDTA-NaOH pH 8 and 10 % (v/v) glycerol using a HiPrep 26/10 desalting column (GE Healthcare). The purified protein used for transport assays was snap frozen in liquid nitrogen and stored at -80 °C. Alternatively, the purification was followed by His$_6$-tag cleavage with 3C protease. BtuF was incubated with 3C protease in a 1:20 molar ratio (3C to BtuF) for 1 hour at °4 in reducing condition (2 mM β-mercaptoethanol). The reaction mix was supplemented with 20 mM imidazole-HCl pH 8 and the cleaved His$_6$-tag and tagged 3C protease were removed by IMAC (reverse Ni-NTA). Tagless BtuF was desalted into 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 10 % (v/v) glycerol for crystallization purposes. Alternatively, the same desalting buffer was used except for HEPES pH 7 instead of Tris buffer to continue with fluorescent labeling. The protein sample and buffers were always kept on ice or at 4 °C.

### 2.3.7 Crystallization of Cbi-bound BtuF

(Tianyu Zhou crystallized the BtuF W66X mutants)

BtuF purification was performed as described previously with some modifications including His$_6$-tag cleavage and periplasmic preparation in case of BtuF W66X mutants performed by
T. Zhou [8, 13]. Additional purification steps using preparative size-exclusion chromatography (SEC) with a superdex 200 10/300 GL column were performed to obtain purer sample. Final buffer composition was 10 mM Tris-HCl pH 8 and 100 mM NaCl. For crystallization purposes, BtuF was concentrated in the presence of 0.2 mM di-cyano-cobinamide (Sigma C3021). The sample was supplemented with approximately 1 mM di-cyano-cobinamide prior to setting up crystallization trays at about 20 mg/ml. The crystallization experiment was performed by sitting drop vapor diffusion technique at 20°C. Depending on the pH of the reservoir condition, HEPES sodium pH 7 or Tris-HCl pH 8.2 and higher, the crystallization drops appeared orange-red or purple corresponding to the mono-cyano- or di-cyano-form of cobinamide. 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) PEG3350) and were optimized in 24-well plates. Orange-red rod-shaped crystals grew to approximately 300 µm in size and were cryoprotected by stepwise increase of glycerol to 25 % (v/v). Mono-cyano-cobinamide was bound in the crystal structures corresponding to the drop and crystal colors.

2.3.8 Data collection and structure determination of Cbi-bound BtuF

(Tianyu Zhou collected the data of the BtuF W66X mutants)

Data collection was performed at the Swiss Light Source (PSI, Villigen) and data processing was done with XDS [14]. Phasing of the wild-type data set was performed by molecular replacement in Phenix using the Cbl-bound BtuF structure (PDB ID 1N2Z) devoid of Cbl and waters as search model. Initial phases for the Cbi-bound BtuF 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 Coot [15, 16]. Additional refinement steps were done with Refmac5 in the CCP4 program suite. PDB file modifications like removing Hydrogens, ANISO records or alternative conformations were performed using the CCP4 program suite [17]. Final figures were prepared using PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).
2.3.9 Fluorescent labeling of BtuF

BtuF was purified as described in 2.3.6 including His<sub>6</sub>-tag cleavage. To prevent oxidation of an introduced cysteine, 5 mM β-mercaptoethanol was used during purification and only removed immediately before labeling. BtuF was labeled with fluorescein-5-maleimide (Thermo scientific 62245) at an engineered cysteine residue (Q145C) in the backbone helix as described previously [18]. In brief, 50 to 100 µM BtuF Q145C (Invitrogen QuickChange) was labeled with a 10-fold molar excess of fluorescein-5-maleimide generating BtuF<sub>fluo</sub>. 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 SEC. Protein concentration was determined using a Bradford assay with 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 pH 7, 200 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 10 % (v/v) glycerol.

2.3.10 Substrate binding assays with BtuF<sub>fluo</sub>

(Tianyu Zhou contributed to the substrate binding assays)

The assay is based on monitoring the ligand-induced change in emission of fluorescein attached to BtuF (BtuF<sub>fluo</sub>). A similar approach has been used to determine the affinity of maltose-binding protein for maltose in the presence of various antibodies [19-21]. 5 nM BtuF<sub>fluo</sub> was incubated with a range of substrate concentrations from 0.02 nM to 33 µM. Binding buffer composition was 50 mM Tris-HCl pH 7.5 and 200 mM NaCl. 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<sub>D</sub> determination according to Y = (B<sub>max</sub> × X)/(K<sub>D</sub> + X). GraphPad Prism version 6.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com".).
2.4 Results and Discussion

2.4.1 $^{14}$C-labeling of cobinamide

To investigate \textit{in vitro} Cbi transport by BtuCD, radiolabeled substrate was required, which was not commercially available. We exploited the fact that CN-Cbi (cyano-aquo-cobinamide) is a stable and inert species and synthesized $^{14}$CN-Cbi by an oxidative reaction using aquo-cob(II)inamide and $^{14}$C-labeled KCN as educts (Fig. 2.4).

![Reaction scheme of $^{14}$CN-Cbi generation (\textit{\textsuperscript{14}C} labeled cyano-aquo-cob(III)inamide).](image)

A 0.85 mol equivalent of K$^{14}$CN 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 $^{14}$CN-Cbi product formation was monitored by recording UV/Vis spectra which clearly showed shifts of the absorption peaks in the range of 300 – 400 nm and 450 – 550 nm (Fig. 2.5). Formation of di-cyano-cobinamide could be ruled out by the absence of an absorption peak at 580 nm. In addition, we analyzed the reaction mix using silica thin layer chromatography, because educt and product can be separated by this method (data not shown).
Figure 2.5: Generation of radioactive cobinamide. A) UV/Vis spectra of mono- and di-cyano-cobinamide. KCN was titrated to 41 nmol aquo-cob(II)inamide using 0, 0.5, 1, 1.5, 2 and 4 mol equivalent of CN (CN to cob(II) ratios 0:1, 0.5:1, 1:1, 1.5:1, 2:1 and 4:1). UV/Vis spectra were recorded 30 min after incubation and at a substrate concentration of 41 µM. The initial substrate aquo-cob(II)inamide is shown in green and the mono- and di-cyano-forms of the product are represented in red and blue, respectively. Di-cyano-cobinamide shows an additional peak around 580 nm, which is absent in the spectrum of aquo-cob(II)inamide or the mono-cyano-form. Note that at a 1:1 mol ratio di-cyano-cobinamide formation starts to occur. B) Analysis of generated \(^{14}\)CN-Cbi by UV/Vis spectroscopy. A 0.85 mol equivalent of \(^{14}\)CN was titrated to 4.8 µmol aquo-cob(II)inamide at 22°C. UV/Vis spectra of the reaction mix diluted to 47 µM cobinamide were recorded 30 to 60 min after each sequential addition of \(^{14}\)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)inamide is shown in brown. Titration steps in between are shown with thin lines.

2.4.2 Orientation of BtuCD in liposomes

For in vitro transport assays, BtuCD was reconstituted into liposomes. Only transporters oriented right-side-in (BtuD in the liposome lumen) can catalyze productive substrate transport (Fig. 2.6A). The right-side-in reconstitution efficiency was determined using a fluorescein-labeled BtuF variant (BtuF\(_{\text{flu}}\)) that was previously shown to be fully functional [18]. Approximately 10 % of BtuCD could associate with BtuF\(_{\text{flu}}\) and thus were oriented right-side-in (Fig. 2.4B-C). This value agreed with the previously determined 7±2 %, which was established by measuring the amount of trapped, non-transported, radio-labeled Cbl associated with BtuCD [22].
**Figure 2.6: Orientation of BtuCD in liposomes.**

A) Schematic of the assay used to determine the amount of BtuCD accessible to BtuF\textsubscript{fluor}. Proteins are displayed in ribbon representation with BtuF in blue and the fluorescent label in green, BtuC in cyan and blue, and BtuD in yellow and red. Only the fraction of BtuCD oriented in the right-side-in (BtuD in liposome lumen) can bind to BtuF\textsubscript{fluor}. ATP and an ATP-regenerating system (ARS) were incorporated into the liposome lumen.

B) Shown is the raw fluorescent signal associated with the liposomes after incubation and removal of excess BtuF\textsubscript{fluor}. The signal of empty liposomes (empty) was subtracted from the proteoliposome signal. WT, wild-type; E159Q, hydrolysis-deficient BtuCD mutant.

C) Illustrated is the accessibility of BtuCD to BtuF\textsubscript{fluor}. Binding of BtuF\textsubscript{fluor} was measured in the presence or absence of ATP. The amount of BtuF\textsubscript{fluor} associated with BtuCD in the presence of ATP was used to correct substrate transport for the fraction of accessible BtuCD.
2.4.3 *In vitro* \(^{14}\)CN-Cbi transport by BtuCD in liposomes

To start the Cbi transport reaction, BtuF previously incubated with \(^{14}\)CN-Cbi was added to BtuCD-containing liposomes (Fig. 2.7 A). All transport rates were corrected for the amount of right-side-in oriented transporters and later also for the background level of Cbi associating with proteoliposomes in the absence of ATP. An initial transport rate of \(0.7 \pm 0.2 \text{ nmol Cbi / mg protein / min}\) was determined (Fig. 2.7 B), with a 2-fold variability in absolute rates depending on the proteoliposome batch. BtuCD-catalyzed Cbi transport was ATP- and BtuF-dependent, as was observed for Cbl transport [22]. The calculated *in vitro* turnover number for Cbi transport was approximately 1.5-fold slower than that of Cbl transport. This makes Cbi a bona fide substrate of *E. coli* BtuCD-F.

![Diagram of in vitro Cbi transport by BtuCD reconstituted into liposomes](image)

**Figure 2.7: In vitro \(^{14}\)CN-Cbi transport by BtuCD in liposomes.** A) Schematic of *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 (ARS) was incorporated into the liposome lumen. B) Cbi transport activity of wild type BtuCD-F. Transport was measured under the following conditions: 4 mg/ml proteoliposomes (~ 0.5 µM total BtuCD), 1 µM BtuF, 15 µM 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.
2.4.4 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 1N2Z) devoid of Cbl as a model (Fig. 2.8 and 2.9). Well-defined electron density in the difference map was observed for bound Cbi as well as the side chain of W66 after initial rigid-body refinement using BtuF devoid of substrate (Fig. 2.9 A). The structure of Cbi-bound BtuF was similar to that of Cbl-bound BtuF (pdb ID 1N4A) with a RMSD of 0.419 Å when using the corrin ring to anchor the superimposition. The six residues Y50, W66, W85, F162, F168 and W196 in the substrate cavity all contribute to Cbi binding, which was previously reported for Cbl-bound BtuF [4, 5]. The only significant structural difference was observed for W66, whose side chain swung approximately 130° around the Cα-Cβ bond towards the inside of the binding pocket, to the location that is occupied by the 5-methyl group of DMB in Cbl-bound BtuF (Fig. 2.9B-C). There was no direct coordination of the Co atom of Cbi by the side chain of W66, unlike the bond between the DMB moiety and the Co atom in Cbl-bound BtuF [4]. Instead, we observed density 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 BtuF [5].

Figure 2.8: Crystal and diffraction pattern of Cbi-bound BtuF. A) Shown is a typical Cbi-bound BtuF crystal grown in 50 mM HEPES sodium pH 7, 1 % (w/v) tryptone, 12 % (w/v) PEG3350. Note the red color of the crystal indicating that the mono-cyano form of cobinamide was bound. B) Displayed is a diffraction image collected from a Cbi-BtuF crystal. Data collection settings were 0.1 s exposure time, 0.1 ° oscillation range and 0.1 filter transmission. Resolution rings are 4.65 Å, 2.44 Å and 1.75 Å.
Figure 2.9: Crystal structure of Cbi-bound BtuF. A) Cbi-bound BtuF model (PDB ID 5M29). BtuF is depicted in green cartoon and cyano-aquo-cobinamide is shown in stick representation (carbon, mangenta; oxygen, red; nitrogen, blue; cobalt, light brown). The N- and C-termini of BtuF are indicated as N and C, respectively. After initial rounds of refinement with omission of the ligand in the model, unambiguous electron density for Cbi was displayed in the Fo-Fc map contoured at ± 3.0 σ (box to the right and viewed from C-lobe). The disordered amino acid residue stretch (217 to 232) is indicated with a dashed black line. B) Comparison of the substrate-binding sites of Cbi-bound BtuF (left box) and Cbl-bound BtuF (right box, PDB ID 1N4A). In Cbi-bound BtuF residue W66 adopts a new rotamer (black arrow) facing the inside of the substrate binding site and filling the space occupied by DMB in Cbl-bound BtuF. The two axial ligands of Cbi, a cyanide and a water molecule, are shown as stick and red sphere, respectively. For Cbi-bound BtuF the same coloring was used as in panel A. Cbl-bound BtuF is illustrated as gray cartoon for the protein part and stick representation for Cbl (carbon, red; oxygen, red; nitrogen, blue; cobalt, light brown; phosphate, yellow). Both substrate-binding sites are shown from the front view similar to panel A. C) Chemical structure of the bigger substrate Cbl illustrating the additional 5,6-dimethylbenzimidazole (DMB) moiety and phosphoribosyl linker, which are absent in the smaller substrate Cbi. The two axial ligands DMB and cyanide are in the α- and β-position, respectively, and coordinated by the central Co atom.
2.4.5 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 (Fig. 2.10). The binding assay was performed with CN-Cbl or di-CN-Cbi and BtuF\textsubscript{fluo}. We found that binding of Cbl or Cbi quenches the fluorescence of the covalently attached fluorescein label, allowing the precise determination of bound substrate (Fig. 2.11). Using this assay, we determined a dissociation constant $K_D$ of Cbl and WT BtuF\textsubscript{fluo} in the low nM range, as reported previously [23]. No substantial changes in affinity for Cbl could be determined for W66 mutants (Fig. 2.11 and table 2.1) except for mutations to charged side chains (arginine or glutamate), which caused a decrease in affinity of about 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 2.1). 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 2.1). The largest change was observed for the W66E and W66A mutations, with $K_D$ values in the low micromolar range. These results indicate that high affinity Cbi binding requires an aromatic, hydrophobic, and bulky residue such as tryptophan or phenylalanine at position 66.

Figure 2.10: BtuF\textsubscript{fluo} preparation. A) Fluorescein labeled BtuF (BtuF\textsubscript{fluo}) was purified by size-exclusion chromatography. Wild-type (WT) BtuF\textsubscript{fluo} showed a monodisperse peak and similar traces were obtained for the mutants. The absorbance at 494 nm detects the co-elution of the label with BtuF. B) Coomassie stained 15% non-reducing SDS-PAGE showed a clean purification for WT BtuF\textsubscript{fluo} (W66) and mutants (W66X, X = A / F / Y / R / E / H / L). In gel fluorescence was detected at an excitation wavelength of 488 nm and an emission wavelength of 526 nm.
Figure 2.11: Substrate binding to WT BtuF<sub>flu</sub> and W66E. A) The substrate-binding assay is based on the quenching of the fluorescence signal of BtuF<sub>flu</sub> in a substrate concentration dependent manner. The residue Q145C in the backbone helix was labeled with fluorescein-5-maleimide (green stick) and W66X (dark blue) was the site of mutagenesis. The model with PDB ID 1N2Z was used to generate the figure. B) Example curves of Cbl and Cbi binding to WT BtuF<sub>flu</sub> and W66E. BtuF<sub>flu</sub> was used at 5 nM and the substrate concentration ranged from 0.02 nM to 33 µM. Indicated are mean and SD (n=3). For determined K<sub>D</sub> values see table 2.1.

Table 2.1: Dissociation constant K<sub>D</sub> values of Cbl and Cbi binding to wild-type BtuF<sub>flu</sub> and W66 mutants. The substrate-binding assay is based on fluorescence quenching of fluorescein labeled BtuF (BtuF<sub>flu</sub>) in a substrate concentration dependent manner. BtuF<sub>flu</sub> was used at 5 nM and substrate concentration was varied from 0.02 nM to 33 µM. Note that K<sub>D</sub> 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 K<sub>D</sub> values are shown in figure 2.10.

<table>
<thead>
<tr>
<th>E.c. BtuF</th>
<th>K&lt;sub&gt;D&lt;/sub&gt; of Cbl, nM</th>
<th>K&lt;sub&gt;D&lt;/sub&gt; of Cbi, nM or µM</th>
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<tr>
<td>WT</td>
<td>9.1 ± 2.6</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>W66F</td>
<td>8.0 ± 1.9</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>W66Y</td>
<td>5.7 ± 1.7</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>W66L</td>
<td>15 ± 2</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>W66H</td>
<td>5.5 ± 1.7</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>W66R</td>
<td>68 ± 13</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>W66A</td>
<td>14 ± 4</td>
<td>1.8 ± 0.8</td>
</tr>
<tr>
<td>W66E</td>
<td>57 ± 15</td>
<td>2.9 ± 0.8</td>
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2.4.6 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 2.2). 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. 2.12). 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 Cbl-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 in the 2Fo-Fc and Fo-Fc omit maps allowed clear modeling of the tyrosine side chain in chain A of the asymmetric unit. Interestingly, the tyrosine side chain of W66Y was also pointing out of the substrate-binding pocket, similar to the W66L structure. In chain B, the side chain was not visible, suggesting flexibility. Extra density was also present near the corrin ring and was modeled as a glycerol molecule, similar to the W66L structure. The side chain orientation of W66L and W66Y can explained the lower Cbi binding affinities of the corresponding BtuF constructs, because these two residues did not seem to contribute to Cbi binding.

The corrin ring of Cbi can bind cyanide either at the α- or β-position. Judged by the red color of the crystals, BtuF-bound Cbi contained only one cyanide group (the di-cyano-form would have been purple). However, it could not be determined unambiguously whether cyanide was bound in the α- or β-position in all BtuF variants. Only for the W66F variant cyanide could be clearly placed at the β-position in both protein chains of the asymmetric unit. In chain A of
the wild-type structure, cyanide was in the α-position of Cbi. In chain B, there was some
density both at the α- and β-positions, and therefore a cyanide group was modeled on both
sides, each with an occupancy of 0.5. For chain A of the W66Y variant, and for chain B of
W66L, bound cyanide was observed in the β-position, while there was no electron density at
the α-position for the second axial ligand. In the other protein chain cyanide could be bound
at either position, and therefore again modeled with an occupancy of 0.5. Thus, the BtuF
substrate-binding site does not seem to have a preference for the α- or β-mono-cyano form of
the substrate, since the cyanide group 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.
Figure 2.12: 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. Note the alternate conformation of one of the hydroxyl groups of glycerol in panel D. 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, magenta; 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 Cα 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).
2.4.7 *In vitro* Cbl and Cbi transport by BtuCD with BtuF W66 mutants

Substrate transport was measured using commercially available $^{57}$Co-Cbl and in-house synthesized $^{14}$CN-Cbi as described before. 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. 2.13). This finding indicates that under the conditions used, W66 is important for high affinity Cbi binding, but not for substrate delivery or transport.

![Figure 2.13: Substrate transport with BtuF W66 mutants and wild-type BtuCD. Shown are the initial transport rates of Cbl and Cbi. Transport was measured under the following conditions: 4 mg/ml proteoliposomes (~0.5 µM total BtuCD), 1 µM BtuF, 2 mM ATP, 15 µM cobinamide or cyanocobalamin. Indicated are mean and S.E.M of the transport rates of Cbl (n=6 for wt, n=9 for mt) or Cbi (n=6).](image)

2.4.7 Discussion

A method to synthesize radiolabeled Cbi was developed and *in vitro* transport assays confirmed that *E. coli* BtuCD-F binds and transports the Cbl precursor Cbi. Wild-type BtuCD-F transported Cbi 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, the X-ray structure of Cbi-bound BtuF was determined. A single residue, W66, was found to have a pivotal role. This residue is located in the binding site and appeared 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 [24]. 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 indicated that W66 was 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 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 (or cluster A) periplasmic SBP [25-27], 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 ligand [28]. 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 moiety [29]. The most drastic change in coprogen-bound FhuD was 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 SBP. For example, DEBP (L-aspartate / L-glutamate binding protein) from Shigella flexneri can bind both aspartate and glutamate while simultaneously discriminating against glutamine and asparagine [30]. 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 affinities [31]. 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 moiety [31, 32]. Specifically, the Cu 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 (or MBP), the periplasmic SBP, can bind maltose, maltotriose, and larger glycans up to maltodecapentaose (maltodextrin with n=15), as well as cyclic glycans, all with similarly high affinities [33, 34]. Structures of MBP bound to maltose, maltotriose and maltotetraose revealed that the solvent accessible amount of the substrate increases with substrate size [35]. 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 MalE [36, 37]. 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 domain [38]. 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 [39]. Furthermore, the size of the amino acid substrate itself affects opening and closure of the substrate-binding site.

It is currently unknown whether other Cbi analogues can serve as substrates for BtuCD-F. However, our findings not only established 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.
Table 2.2: Crystallographic table with data collection and refinement statistics. Highest-resolution shell values are shown in parentheses. The correlation coefficient is abbreviated CC [40].
References

3. Probing the BtuCD translocation pathway by mutagenesis and functional studies

3.1 Abstract

BtuCD-F is responsible for the uptake of cyanocobalamin and cobinamide into the cytoplasm of *Escherichia coli*. Cobinamide is lacking the 5,6-dimethylbenzimidazole and phosphoribosyl linker of cyanocobalamin and is therefore about a third smaller. The newly generated $^{14}$C-labeled cobinamide described in chapter 2 made it possible to measure *in vitro* transport and compare it to $^{57}$Co-cyanocobalamin. The transport rates were similar; therefore, if and how the substrate translocation pathway senses the two structurally distinct substrates was unknown. Semi-conserved residues at specific positions within the translocation pathway were mutated to assess the effect on substrate translocation. The mutants were purified and reconstituted into liposomes to measure ATPase activity and *in vitro* transport. They showed similar ATPase activities as the wild-type and were able to bind BtuF. *In vitro* cobinamide transport tolerated more modifications to the translocation pathway than cyanocobalamin, except for mutations to histidines, which abolished its transport. Methionines at the entrance of the translocation pathway were crucial for cyanocobalamin transport. The presence of bulky residues halfway through the translocation pathway imposed size restrictions on cyanocobalamin transport, but much less on cobinamide. These results indicated that despite the lack of a substrate-binding site, the translocation pathway is not inert, but senses the size and chemical properties of the transported substrate and thus contributes to substrate specificity.

3.2 Introduction

To probe the effect of size and side chain chemistry on substrate transport, mutations were introduced at strategic positions within the substrate translocation pathway. The mutants were designed based on the previously reported AMPPNP-bound BtuCD-F structure [1]. The assembled transporter, BtuC$_2$D$_2$-F, was composed of two BtuC subunits forming the TMDs
and two BtuD subunits building the NBDs (Fig. 3.1A). The BtuC subunits contain the substrate translocation pathway and the BtuD subunits bind and hydrolyze ATP to power substrate translocation. The structure of AMPPNP-bound BtuCD-F revealed a central cavity within the translocation pathway, big enough to harbor a Cbl molecule with minor steric clash (Fig. 3.1A-B) [1]. The cavity was closed to both sides, at the top sealed by a periplasmic gate and at the bottom by a cytoplasmic gate II. The interior of the cavity was lined with residues of TM3, TM5, and helix H5a, and contained a methionine patch, 4 methionines per BtuC subunit, two of which pointed directly into the translocation cavity (M176 and M179).

The mutations were designed based on the observed cavity. The first set of mutations was located at the narrow entrance of the cavity close to the periplasmic gate (M176 and M179 in H5a). These methionine residues were mutated to alanines to expand the cavity entrance, or to histidines to change its chemical properties. The mutations were studied individually and combined to a double mutant. The position of the second set of mutations was halfway through the cavity (S157 in TM5) with a distance of 16 Å between the two residues. The serine was replaced by a tryptophan or phenylalanine to assay the effect of constriction. On the other hand, the chemical properties were modified by substitution with histidine. Since the assembled transporter is composed of two BtuC subunits, introduction of one mutation results in a total of two. Nine mutants were investigated for their effect on ATP hydrolysis, substrate transport and complex formation with BtuF. The mutated residues were semi-conserved between BtuC proteins of different organisms and comparison to other type II importers showed that only S157 was also present in Haemophilus influenzae HI1471 (Fig. 3.1C).
Figure 3.1: Position of the cavity mutants. A) Crystal structure of AMPPNP-bound BtuCD-F (PDB ID 4FI3). The model is shown in cartoon representation with BtuF in green, BtuC in cyan and blue, and BtuD in yellow and pink. The cavity within the substrate translocation pathway formed by the two BtuC subunits is illustrated as a grey surface representation. The dimensions of the cavity are approximately 25 Å in the vertical direction (M176 to L85) and 16 Å in the horizontal direction (S157 to S157’). Prime indicates the second BtuC subunit in the dimer. The program HOLLOW was used to generate the surface of the cavity. Mutated residues are shown with yellow spheres. B) Close-up view of the cavity. Illustrated are the locations of M176 and M179 at the entrance of the cavity in helix H5a and S157 further down halfway through the cavity in helix TM5. The sites of mutagenesis are displayed as yellow spheres. Note that one mutation in BtuC results in two mutations within the assembled dimer. The distances between the Cα atoms of the mutated residues in the two BtuC subunits are 16 Å (S157), 10 Å (M176) and 14 Å (M179). C) Protein sequence alignment of TM5 and H5a. Shown is BtuC aligned to type II ABC importers with experimentally confirmed function (top) and to BtuCs from different organisms (bottom). E. c., Escherichia coli; H. i., Haemophilus influenzae; Y. p., Yersinia pestis; P. p., Photobacterium damselaiae subs. piscicida; S. t., Salmonella enterica serovar Typhimurium; P. a., Pantoea ananatis; K. r., Klebsiella pneumoniae subs. rhinoscleromatis; S. f., Shigella flexneri; V. r., Vibrio rubber; H. t., Halorhabdus tiamatea; H. p., Halolamina petagica.
3.3 Experimental procedures

Expression and purification of BtuF and BtuCD, reconstitution of BtuCD into liposomes, proteoliposome characterization, and in vitro transport assays with liposomes are described in chapter 2.

3.3.1 BtuCD-F complex purification in detergent

BtuCD-F complex formation was performed as described previously [1, 2]. Purified BtuCD and BtuF were mixed using a 3-fold molar excess of BtuF. The buffer contained 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 25 mM imidazole-HCl pH 8, 0.1 % (w/v) LDAO and 0.01 % (v/v) C₁₂E₈ Anagrade. The sample was loaded on a Ni-NTA resin equilibrated with 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 25 mM imidazole-HCl pH 8 and 0.01 % (v/v) C₁₂E₈ and washed with the same buffer for detergent exchange to 0.01 % (v/v) C₁₂E₈. The protein was eluted with 200 mM imidazole-HCl pH 8 and concentrated (100 kDa cutoff) to 500 μl. The BtuCD-F complex was purified by preparative size-exclusion chromatography using a superdex 200 10/300 GL column equilibrated with 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 0.01 % (v/v) C₁₂E₈.

3.3.2 Production of cavity mutants

The cavity mutants were generated using standard molecular biology cloning techniques. The mutations were introduced into cys-less wild-type BtuCD [3] either by site-directed mutagenesis or insertion of gene fragments containing the mutation (gBlocks, Integrated DNA technologies). For the gBlock-based cloning, a synthetic btuC gene (synbtuC) with silent restriction sites PstI and SmaI was generated including codon optimization for expression in E. coli (GeneArt). Gene fragments containing M176A, M176A/M179A and M176H/M179H were inserted into synbtuC with PstI and SmaI and subcloned into the BtuCD expression vector with NcoI and NheI. Introduced mutations were validated by DNA sequencing (Microsynth) and synbtuC was sub-cloned into the BtuCD expression vector.
Expression, purification and reconstitution of the BtuCD cavity mutants were performed as described for the wild-type in chapter 2.

Figure 3.2: BtuCD expression construct with synthetic btuC (synBh). A synthetic btuC gene with silent restriction sites PstI and SmaI was generated for gBlock-based cloning.

### 3.3.3 ATPase activity assay

ATP hydrolysis rates were determined using a modified molybdate method for inorganic phosphate determination [4]. The ATPase activity was determined at RT, and the reaction contained 1 mM ATP, 5 mM MgCl₂, 20 mg/ml liposomes (0.23 – 0.35 mg/ml or 0.18 - 2.7 µM total BtuCD) and the buffer composition was 10 mM Tris-HCl pH 7.5 and 100 mM NaCl. Time points were taken within 25 min by stopping 50 µl of the reaction with the addition of 50 µl of 12 % (w/v) SDS. Measurements were performed within the linear range of ATP hydrolysis and rates were determined using linear regression in Graphpad Prism 7.

### 3.3.4 Spheroplast preparation

*E. coli* BL21 (DE3) Gold cells were individually transformed with plasmids encoding wild-type BtuCD, a cavity mutant or the hydrolysis-deficient mutant BtuCD<sub>E159Q</sub>. Cells were grown in TB medium supplemented with 1 % (w/v) glucose and 100 µg/ml ampicillin until the 40 ml culture reached OD<sub>600</sub> of 1. Protein expression was induced with 0.25 mM IPTG for 1 hour at 37 °C. Cells were harvested by centrifugation in 50 ml falcons (3500 g, 30 min, 4°C) and the pellet was stored at -80 °C. Each construct was expressed in three independent cultures for triplicate measurements. Spheroplasts were prepared using a modified version of a procedure described previously [5, 6]. Approximately 0.2 – 0.3 g cell pellet was resuspended and washed in 7 - 10 ml of 0.2 M Tris-HCl pH 8 and finally resuspended in 1 - 2
ml. An equal volume of 1 M sucrose in 0.2 M Tris-HCl pH 8 was added followed by 50 µg/ml lysozyme and 0.5 mM EDTA-NaOH pH 8. Samples were diluted with 4 ml cold ddH2O followed by the addition of MgCl2 and DNase I to final concentration of 3 mM and 20 µg/ml, respectively. To remove buffer components, the spheroplasts were pelleted by centrifugation (3500 g, 30 min, 4°C) and resuspended at a final concentration of 0.16 g spheroplasts/ml in 0.25 M sucrose, 0.05 M Tris-HCl pH 8 and 3 mM MgCl2. All steps were performed on ice. Spheroplasts formation was confirmed under a microscope.

3.3.5 BtuCD expression level in spheroplasts.

To determine the amount of functionally expressed BtuCD in the spheroplasts, 4 µM of fluorescently labeled BtuF (BtuF_fluo) was incubated with an equal volume of 0.16 g/ml spheroplasts and incubated for 30 min at RT. Spheroplasts were pelleted to remove unbound excess BtuF_fluo, and the pellet was washed with a buffer containing 0.25 M sucrose, 0.05 M Tris-HCl pH 8 and 3 mM MgCl2. Spheroplasts with bound BtuF_fluo were resuspended at 0.08 g/ml and dissolved in 1.7 % (w/v) SDS. The fluorescence associated with the samples was measured with a plate reader using an excitation wavelength of 485 nm and emission was recorded at 516 nm. The amount of bound BtuF_fluo was determined by using a BtuF_fluo calibration curve.

3.3.6 Substrate transport assays with E. coli spheroplasts

Substrate-loaded BtuF and spheroplasts containing overexpressed BtuCD were incubated at RT for 30 min prior to reaction start. Time points were taken by diluting 50 µl of the ration in 300 µl ice cold stop buffer containing 0.25 M sucrose, 0.05 M Tris-HCl pH 8, 3 mM MgCl2, 8 % (w/v) PEG6000 and 100 µM cold Cbl. The stopped reaction mix was transferred to a manifold filtration system (Millipore MSFBN6B) and washed twice with 200 µl cold stop buffer. The concentrations used for the transport assay were 5 µM BtuF, 15 µM substrate (14CN-Cbi or 57Co-Cbl), 0.08 g/ml spheroplasts (0.2 – 0.5 µM BtuCD varying for mutants) and the buffer composition was 0.25 M sucrose, 0.05 M Tris-HCl pH 8 and 3 mM MgCl2. The radioactivity measurement procedure and rate determination were the same as described for substrate transport in liposomes described in chapter 2. The determined transport activities were normalized according to BtuCD expression level.
3.4 Results and Discussion

3.4.1 Production of cavity mutants

A synthetic \( btuC \) gene with silent restriction sites was generated for the cloning of three cavity mutants, which could not be obtained by site-directed mutagenesis. The wild-type expression construct with the synthetic \( btuC \) gene, synBh, had an expression yield of 0.5 mg protein / g cells, which was 50 % of the yield obtained for the Bh expression construct without a synthetic \( btuC \) gene (described on page 28, Fig. 2.2). This was surprising, since the synthetic BtuC gene was codon optimized for expression in \( E. coli \) cells. However, size-exclusion chromatography (SEC) analysis showed a stable and monodisperse peak of the purified transporter. SDS-PAGE analysis of detergent-purified and in liposome-reconstituted protein confirmed the presence of BtuC and BtuD (Fig. 3.3A). All cavity mutants showed similar purification yields as the corresponding wild-type. The stability of the mutants was assessed by the monodispersity of the sample using SEC analysis. All cavity mutants were stable and showed a monodispersed peak, and were successfully reconstituted into liposomes (Fig. 3.3B). BtuF complex formation was analyzed for the M176A/M179A using SEC analysis, which showed co-elution of BtuF with the mutant transporter.
Figure 3.3: Purification and reconstitution of cavity mutants. Shown are representative size-exclusion chromatography (SEC) profiles and 15 % SDS-PAGE analysis for WT synBtuCD (A) and BtuC_{M176A/M179A} (B). 500 µl of an approximately 0.8 mg/ml protein sample was injected on a superdex 200 10/300 GL column equilibrated with 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 0.1 % (w/v) LDAO. Purified protein in detergent (D) or reconstituted into liposomes (L) are indicated. Similar results were obtained for all mutants. The lane in panel B labeled with a red asterix corresponds to complex formation with BtuF and isolation by SEC in 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 0.01% (v/v) C_{12}E_{8}.

3.4.2 ATPase activity of the cavity mutants in liposomes

The integrity of BtuCD mutants reconstituted into liposomes was evaluated by measuring the ATPase activity, using the inside-out oriented transporters (BtuD outside of the liposomes; Fig. 3.4A). The ATPase rate of wild-type (WT) BtuCD was 42.6 ± 1.3 nmol P / mg protein / min (Fig. 3.4B). Most of the cavity mutants showed similar rates, except for M179A and the histidine mutants at the cavity entrance, which had a 1.5 – 2 - fold higher activity. These mutations might facilitate nucleotide binding and/or hydrolysis by changing the BtuCD dynamics. The ATP hydrolysis activity of all mutants confirmed the functional integrity of the cavity mutants reconstituted into liposomes.
Figure 3.4: ATPase activity of the cavity mutants in liposomes. A) Schematic of the ATPase activity assay. BtuCD transporters insert into liposomes either in the right-side-in (BtuD in the liposome lumen) or inside-out (BtuD facing the outside) orientation. The inside-out oriented transporters were used to measure ATP hydrolysis, thus ATP was added to the outside to initiate the hydrolysis reaction measured at RT. The two BtuC subunits are shown in blue and cyan, and the two BtuD subunits in red and yellow. The BtuCD model with PDB ID 1L7V was used to generate the figure. B) ATPase rates. The ATPase activity was measured using 20 mg/ml liposomes (0.23 – 0.35 mg/ml or 1.8 - 2.7 µM total BtuCD), 1 mM ATP, 5 mM MgCl₂ and the buffer composition was 10 mM Tris-HCl pH 7.5 and 100 mM NaCl. Shown are mean and SEM for n = 3.

3.4.3 Effect of the cavity mutants on substrate transport in liposomes

To assay the role of distinct residues within the translocation pathway, a mutagenesis study was performed. The cavity mutants were analyzed for their effect on transport of the structurally related but differently sized substrates Cbl and Cbi (Fig. 3.5A). In the assay setup, only the transporters oriented right-side-in (BtuD in liposome lumen) were accessible for substrate-loaded BtuF added from the outside and yield successful transport (Fig. 3.5B). About 10 % of reconstituted BtuCD was accessible and could be used to measure transport activity (see chapter 2). ATP and an ATP-regenerating system (ARS) were incorporated into the liposome lumen to keep a constant ATP concentration within the time course of the
experiment. To initiate the transport reaction, substrate-loaded BtuF was added to the BtuCD containing liposomes.

Two residues at the entrance of the translocation cavity, M176 and M179, were mutated to alanine to assay the effect of reduced restriction on substrate transport. Two single mutants and a double mutant M176A/M179A with the combined mutations were generated. Cbi transport was slightly reduced by M179A (1.7 ± 0.2 nmol Cbi / mg protein / min) compared to the WT activity (2.2 ± 0.1 nmol Cbi / mg protein / min) and M176A showed a 3-fold rate decrease (Fig. 3.5C). The double mutant M176A/M179A further decreased the Cbi transport activity to a rate only 2-fold higher than the unspecific signal measured by omission of ATP (0.24 ± 0.04 nmol Cbi / mg protein / min). The single mutations M176A or M179A reduced Cbl transport 4-fold compared to the WT activity (3.2 ± 0.5 nmol Cbl / mg protein / min) and the double mutant abolished it (Fig. 3.5D).

To assay the effect of altered side chain chemistry, M176 and M179 were also mutated to histidines. The two single substitutions and the double mutant had a detrimental effect on Cbi and Cbl transport. A remaining Cbl transport activity could only be measured for M179H, which was about 4-fold less than the WT activity (Fig. 3.5D). Cbi transport was neither detected for one of the single mutants nor for the double mutant (Fig. 3.5C). A similar effect was observed for the second site of mutagenesis, S157, located halfway through the translocation pathway. S157H completely blocked Cbi transport and a 5-fold reduced activity compared to the WT could be measured for Cbl.

To assay the effect of restrictions, S157 was also substituted with tryptophan or phenylalanine. S157W and S157F reduced Cbi transport 2- and 4-fold compared to WT, but Cbl transport was abolished by introduction of the large side chains (Fig. 3.5C-D). Because Cbi did not contain the DMB and phosphoribosyl linker, there was still space for the larger side chains.
Figure 3.5: Substrate transport by the cavity mutants in liposomes. A) Chemical structures of cyanocobalamin (Cbl) and cyano-cobinamide (CN-Cbi). Since Cbi is missing the 5,6-dimethylbenzimidazole (DMB) moiety and phosphoribosyl linker, it is considerably smaller than Cbl. Note that the cyanide group of cobinamide can be either at the α- or β-side of the corrin ring with a water molecule on the other side (R = CN or H₂O). The molecular weights are 1355.38 g/mol for Cbl and 1035.15 g/mol for CN-Cbi. B) Schematic of in vitro substrate transport in liposomes. Substrate-loaded BtuF is added from the outside of the liposomes and can only bind to right-side-in (BtuD in liposome lumen) oriented BtuCD for successful substrate transport into the liposomes. An ATP regeneration-system (ARS) was incorporated into the liposome lumen for a constant ATP concentration during the experiment (Adapted from [7]). The BtuCD model (PDB ID 1L7V) and BtuF model (PDB ID 5M29) were used to generate the figure. C-D) In vitro substrate transport rates of Cbi (C) and Cbl (D). Transport was measured under the following conditions: 4 mg/ml liposomes (0.36 – 0.54 µM total BtuCD), 1 µM BtuF, 15 µM Cbi or Cbl, and 2 mM luminal ATP. Unspecific interaction of the radioligand was determined by omission of luminal ATP and shown for WT BtuCD (dashed line). Indicated are mean and SEM for n ≥ 3.

Cbi transport tolerated more modifications to the translocation pathway than Cbl, especially in terms of restrictions. Alteration of side chain chemistry by mutation to histidine, however,
had a detrimental effect on Cbi translocation. The Cbi-histidine interaction presumably trapped the substrate within the translocation pathway, and thus blocked its transport. Observations in crystal structures showed that histidine side chains can interact with Cbi by coordinating with the central Co atom (unpublished work of V. Korkhov and J. Bloch), reminiscent of the cobalamin interaction with transcobalamin [8].

The methionine patch at the entrance of the translocation pathway seemed to facilitate especially Cbl transport, since substitution to alanine or histidine were not tolerated. The decreased transport activity of the double mutant M176A/M179A was remarkable, since reduced restriction was expected to facilitate substrate translocation. BtuF binding and ATP hydrolysis were not affected by these mutations. The observed effects were likely due to the interaction of M176 and M179 with the substrate, facilitating its translocation. An alternative explanation is that the methionine patch ensured the integrity of the cavity by functioning as a spacer. Removal of the methionines results in a ‘collapsed’ translocation pathway, which did not directly influence BtuF binding or ATP hydrolysis. In this case, the smaller substrate Cbi could still pass through the collapsed cavity entrance, but not the bigger substrate Cbl. Attempts to visualize the potentially collapsed translocation cavity of the M176A/M179A mutant yielded crystals diffracting to about 7 Å, which was too low for structure determination. Several trials to increase the resolution were not successful. The difficulties in obtaining well diffracting crystals of this construct might be in line with the collapsed cavity entrance having a destabilizing effect on the transporter.

In summary, wild-type BtuCD transported Cbl and Cbi with similar transport rates, despite their difference. Nevertheless, mutations at specific positions within the translocation pathway had distinct effects on the transport of the two substrates. This indicates that the translocation pathway is not just an inert hole for substrate passage, but contributes to substrate specificity by screening for size. Similar studies have been performed for the Yersinia pestis heme uptake system HmuUV-T, an ABC type II importer related to BtuCD-F. Two conserved residues within the translocation pathway were investigated, R176 at the entrance and G164 inside. R176 was important for complex formation with the SBP HmuT, and G164 had a role in screening the substrate size [9]. The authors concluded that substrate selectivity is ensured by a tight fit of the substrate, rather than a high-affinity binding pocket. A tight fit mechanism was probably also the case for Cbl transport by BtuCD, but not for Cbi translocation. Cbl binding experiments with BtuCD-F in detergent confirmed that there was no classical
substrate-binding site within the translocation cavity [10]. Cbl could only be trapped in liposomes when BtuCD-F was stabilized in the nucleotide-bound state [1]. The cavity observed in the nucleotide-bound BtuCD-F structure was claimed to be a low affinity, intermediate translocation chamber. Accordingly, recent molecular dynamics simulations suggested a non-specific binding mode of Cbl with the translocation cavity through weak and variable hydrogen bonds and hydrophobic interactions involving also S157, M176 and M179 [11]. Therefore, the translocation pathway does not only screen for substrate size but some residues like the methionine patch interact with the translocated substrate, despite the lack of a classical substrate-binding site.

A substrate-binding site within the translocation pathway has been described for the maltose/maltodextrin uptake system MalFGK₂-MBP. The transporter was captured in an outward-facing state with AMPPNP, and maltohexaose was bound to MalF, one of the two TMDs [12]. Unlike the substrate cavity in BtuCD-F, which was closed to the periplasm and BtuF, a continuous cavity between MBP and MalF was observed. The presence of a substrate-binding site allowed for the open translocation pathway in MBP-MalFGK₂. The absence of a specific binding site in BtuCD-F requires the sealing of the translocation cavity to the periplasm to ensure unidirectional transport.

### 3.4.4 Substrate transport in spheroplasts

To screen the effect of BtuCD mutants in a high throughput manner, a spheroplast-based transport assay was developed. The spheroplasts contained overexpressed BtuCD. This method did not require purification and reconstitution of the individual mutants and was therefore more suitable for screening several mutants. The procedure for spheroplast formation was confirmed under a microscope (Fig. 3.6).
Figure 3.6: Spheroplast formation. The procedure for spheroplast formation was validated under a DIC microscope (Axiovert 200M). Shown are the rod-shaped *E. coli* cells before treatment (A) and the spherical shape after the treatment (B).

BtuCD expression levels in spheroplasts were determined by the amount of fluorescently labeled BtuF (BtuF\textsubscript{fluor}) associating with the spheroplasts (Fig. 3.7A-B). The mutants had similar BtuCD expression levels except for M176A, M176A/M179A and M176H/M179H, which showed approximately 50% of the wild-type. Cbl transport assays with spheroplasts containing over-expressed cavity mutants were performed and compared to the results obtained in liposomes, showing similar results of the two methods (Fig. 3.7C). Small differences were observed for S157H, M176A/M179A, and M179H showing slightly higher or lower activities. The hydrolysis-deficient BtuCD mutant, E159Q in BtuD \cite{2}, was used as a negative control to ensure the measuring of BtuCD-catalyzed transport. Cbl transport in spheroplast was only determined for cells expressing wild-type BtuCD or E159Q and showed that this assay also worked for detecting Cbi transport (Fig. 3.7D).
Figure 3.7: Substrate transport by E. coli spheroplasts. A) Schematic representation of the transport assay. Substrate-loaded BtuF is added to the spheroplasts to initiate the transport reaction measured at RT. The spheroplasts contained wild-type (WT) BtuCD, a cavity mutant or the hydrolysis-deficient BtuD mutant E159Q as negative control. The BtuCD expression level was determined by the amount of fluorescently labeled BtuF (BtuF\textsubscript{fluor}) associated with the spheroplasts. B) BtuCD expression level in spheroplasts. Spheroplasts of uninduced E. coli cells containing wild-type (WT) BtuCD plasmid were used as negative control. 2 µM BtuF\textsubscript{fluor} was incubated with 0.08 g/ml spheroplasts in 0.25 M sucrose, 0.05 M Tris-HCl pH 8, and 3 mM MgCl\textsubscript{2}. Excess unbound BtuF\textsubscript{fluor} was removed. The transport rates were corrected for the BtuCD expression level. C) Cyanocobalamin transport by spheroplasts. Transport rates were determined using 5 µM BtuF, 15 µM 57\textsuperscript{Co-Cbl}, 0.08 g/ml spheroplasts (0.2 – 0.6 µM BtuCD) in 0.25 M sucrose, 0.05 M Tris-HCl pH 8 and 3 mM MgCl\textsubscript{2}. D) Cobinamide transport by spheroplasts. Transport rates were determined using the same conditions as indicated for Cbl transport except for 15 µM 14\textsuperscript{CN-Cbi}. 
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The reproducibility of the results obtained with liposomes made the transport assays with spheroplasts a valid method for functional investigations of BtuCD mutants. The spheroplast-based transport assay did not require purification of the protein, its reconstitution into liposomes and their characterization. This made the spheroplast-based transport assay suitable for high throughput screening of BtuCD mutants with altered functional activities regarding BtuF binding or substrate transport. Since the transporter was not purified, functional effects from purifying and reconstituting the protein could be excluded. Furthermore, spheroplasts provided a more native like environment compared to BtuCD reconstituted into liposomes.

References

4. Structural basis of BtuF inhibition by conformational nanobodies

A manuscript describing this study is currently under revision with Sci Rep. The manuscript is entitled "Structural basis of nanobody-mediated blocking of BtuF, the cognate substrate-binding protein of the E. coli vitamin B12 transporter BtuCD." by Mireku SA and Locher KP.

4.1 Abstract

Bacterial ABC importers catalyze the uptake of essential nutrients including transition metals and metal-containing co-factors. IgG-based antibody targeting of the substrate-binding protein required for Mn(II) uptake has been reported to result in transport inhibition and may offer avenues for developing antimicrobial agents. The possibility of using nanobodies to inhibit ABC importers was explored using the vitamin B12 transporter of Escherichia coli, BtuCD-F, as a model system. Nanobodies against its substrate-binding protein BtuF were generated and six nanobodies were isolated that decreased the affinity of BtuF for B12. This observation indicated a nanobody-binding site that is in competition with that of B12. We observed a reduction of in vitro transport activity of BtuCD-F when an excess of nanobody over B12 was used. The structure of BtuF in complex with the most effective nanobody revealed the molecular basis of its inhibitory function. Nb9 inserted its CDR3 loop into the substrate-binding pocket of BtuF, preventing simultaneous B12 binding and BtuCD-F complex formation. Our data demonstrate that nanobodies can mediate ABC importer inhibition and may therefore provide an opportunity for novel antibiotic strategies.

4.2 Introduction

ATP-binding cassette (ABC) importers are multi-subunit membrane protein complexes mediating the uptake of essential nutrients [1]. They contain a soluble or membrane-anchored substrate binding protein (SBP) that recognizes the substrate and delivers it to the transporter located in the cytoplasmic membrane [2]. Recently, an approach to inhibit transport by an ABC importer was established by blocking the interaction of the SBP with the transporter using a Fab fragment of an IgG antibody that specifically bound to the SBP and thus
restricted the interaction with the transporter by steric hindrance. This study was performed with the *Staphylococcus aureus* SBP MntC, which is part of the transporter system responsible for the uptake of the essential nutrient Mn(II) [3]. It was hypothesized that nanobodies, single chain variable domain antibody fragment derived from heavy chain only antibodies of camelids, might be able to accomplish similar blocking [4]. This would offer additional possibilities in developing novel antibiotic strategies, because nanobodies are less immunogenic and smaller than antibodies, thus offering certain advantages for therapeutic approaches.

The ABC importer BtuCD-F catalyzes vitamin B12 (cyanocobalamin or Cbl) and cobinamide uptake into the cytoplasm of *Escherichia coli* [5, 6]. Like other members of the ABC transporter super-family, BtuCD-F couples the energy of ATP binding and hydrolysis to substrate translocation across a lipid bilayer [7]. ABC transporters share a common architecture of two transmembrane domains and two nucleotide binding domains (NBDs) [8, 9], but large scale mechanistic diversity has been observed [10]. In BtuCD-F, the BtuC subunits are membrane-bound, the BtuD subunits are the NBDs [11], while BtuF is the periplasmic SBP [12-14]. Crystal structures of BtuCD-F representing distinct states of the transport cycle and extensive functional studies have provided a detailed description of the transport mechanism [15, 16]. Because BtuCD-F is a well characterized transporter, it was suitable as a model to study functional inhibition.

To explore the functional inhibition by blocking the SBP with a nanobody, BtuF-specific nanobodies were generated and their effects on substrate binding and transport were investigated. A particularly promising, inhibitory nanobody was singled out and its structure in complex with BtuF revealed the molecular basis of inhibition. Our data showed that a nanobody, which is much smaller than a Fab fragment, could also mediate inhibition by competing with substrate binding and transport.
4.3 Experimental procedures

4.3.1 BtuF purification and biotinylation

BtuF for the pull-down, crystallization, binding and transport assays, was prepared as described previously and in chapter 2 [6, 15]. The Avi-tagged BtuF construct for the panning was cloned by insertion of a C-terminal Avi-tag between the BtuF gene and the 3C cleavable His$_6$-tag. The construct was expressed and purified as described previously [6]. Biotinylation was performed according to manufacturer’s instructions (Avidity, protocol for BirA enzyme). BirA was added to BtuF in a 1:2 mass ratio and the buffer contained 10 mM MgAcetate, 10 mM ATP, 50 μM Biotin, 10 mM Tris-HCl pH 8 and 100 mM NaCl. The biotinylation reaction was carried out overnight at 4 °C and His-tagged BirA was removed by IMAC. The sample was purified by size-exclusion chromatography with a superdex 200 10/300 GL column. The final buffer was composed of 10 mM Tris-HCl pH 8, 100 mM NaCl and 10 % (v/v) glycerol. Biotinylated BtuF was snap frozen in liquid nitrogen and stored at – 80 °C.

4.3.2 Generation of nanobodies against BtuF

Nanobodies were produced following a modified version of the protocols established in the Steyaert [17] and Dutzler laboratories. In brief, an alpaca was injected with 200 - 300 μg of B12-bound BtuF for the generation of heavy chain only antibodies. Four injections were performed each in an interval of two weeks, and the specific immune response was analyzed by ELISA (by Sasa Stefanic). 1½ weeks after the last injection, 50 ml blood was collected for subsequent lymphocyte preparation using Histopaque-1077 (Sigma 10771) and ACCUSPIN tubes (Sigma A2055). Isolation of the total RNA was performed with the RNeasy Mini Kit according to manufacturer’s procedure (Quiagen, 74104). The cDNA was prepared by reverse transcription and the antibody fragments were amplified by PCR using primers described previously [18, 19]. The PCR products corresponding to the fragment derived from the heavy chain only antibody were isolated by preparative agarose gel and purified with a Gel Extraction Kit (Qiagen 28706). The variable domain of the heavy chain only antibody (VHH) sequences were further amplified by a second PCR and FX cloning sites were introduced to insert the VHH DNA into the phagemid vector for phage display [20]. Electrocompetent TG1 cells were transformed with the VHH DNA library for storage and further preparation of the phage particles. For phage display, biotinylated BtuF was immobilized on a neutravidin
coated plate (Thermo Scientific, 15507) and omission of protein or immobilized transcobalamin were used as controls to quantify enrichment. The phage library was panned against apo- and B12-bound BtuF by phage display, and two rounds of panning were necessary to detect enrichment. A 96-well plate with individual clones of the enriched sub-libraries was sent for DNA sequencing (Microsynth).

### 4.3.3 Expression and purification of nanobodies

E. coli WK6 cells were transformed with plasmids purified from TG1 cells by standard MiniPrep procedure. Expression cultures were inoculated with a pre-culture, and cells were grown at 37 °C and 190 rpm in Terrific Broth (TB) medium containing 100 µg / ml ampicillin, 1 % (w/v) glucose and 2 mM MgCl₂ until an OD₆₀₀ of ~ 0.7 was reached. The temperature was lowered to 25 °C and protein expression was induced with 1 mM IPTG overnight. Cells were harvested by centrifugation, and the nanobodies were extracted from the periplasm. Therefore, cells were resuspended in TES buffer composed of 0.2 M Tris-HCl pH 8, 0.5 mM EDTA and 0.5 M sucrose, and incubated on a rotating wheel for 1 hour at 4 °C. The cell suspension was diluted with double the volume of TES/4, incubated for another hour at 4 °C and centrifuged to isolate the supernatant containing the periplasmic extract. His-tagged nanobodies were purified by IMAC and desalted with PD10 columns following the manufacturer’s instructions (GE healthcare). The storage buffer contained 250 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.5 mM EDTA-NaOH pH 8 and 10 % (v/v) glycerol.

### 4.3.4 Pull-down assay

His-tagged nanobodies were immobilized on Ni-NTA beads and incubated with 1.5-fold molar excess of tagless BtuF for 1.5 hours at 4 °C. Bound protein was separated from excess BtuF by a quick spin and washed with 20 column volumes of wash buffer composed of 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 10 % (v/v) glycerol and 20 mM imidazole-HCl pH 8. Bound protein was eluted with 500 mM imidazole and analyzed by SDS-PAGE.
4.3.5 Substrate binding assay

The substrate binding assay is based on ligand-induced fluorescence quenching of fluorescently labeled BtuF (BtuF_{fluoro}) and was described previously [6]. 5 nM BtuF_{fluoro} was incubated with 5 µM nanobody for 1 hour prior to cyanocobalamin addition ranging from 0.06 nM to 3.7 µM for another hour. The fluorescence signal was detected with 485 nm for excitation and 516 nm for emission. Increased fluorescence quenching occurred with higher substrate concentration, and the normalized ∆ fluorescence in percent was plotted against substrate concentration. For apparent $K_D$ determination, the data was fit to one site – specific binding according to $Y = (B_{max} \times X)/(K_D + X)$ using GraphPad Prism (GraphPad Prism version 7 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com”). Experiments were performed at 10 °C and the buffer composition was 50 mM Tris-HCl pH 7.5 and 200 mM NaCl.

4.3.6 Spheroplast preparation

E. coli BL21 (DE3) Gold cells were transformed with plasmids encoding cysteine free wild-type BtuCD or a hydrolysis-deficient mutant BtuCD_{E159Q}. Cells were grown in TB medium supplemented with 1 % (w/v) glucose and 100 µg/ml ampicillin until the culture reached OD$_{600}$ of 1. Protein expression was induced with 0.25 mM IPTG for 1 hour at 37 °C. Cells were harvested by centrifugation and the pellet was stored at -80 °C. To prepare spheroplasts [21, 22], about 0.2 g cell pellet was washed with 10 ml of 0.2 M Tris-HCl pH 8 and finally resuspended in 2 ml. An equal volume of 1 M sucrose in 0.2 M Tris-HCl pH 8 was added, followed by addition of lysozyme and EDTA to final concentrations of 50 µg/ml and 0.5 mM. Samples were diluted with an equal volume of cold ddH$_2$O and supplemented with MgCl$_2$ and DNase I to final concentrations of 3 mM and 20 µg/ml. To remove buffer components, the spheroplasts were pelleted by centrifugation and resuspended at 0.16 g spheroplasts/ml in 0.25 M sucrose, 0.05 M Tris-HCl pH 8 and 3 mM MgCl$_2$. All procedures were performed on ice and spins were done at 4°C.

4.3.7 BtuCD concentration in spheroplasts

To determine the amount of functionally expressed BtuCD in the spheroplasts, 4 µM BtuF_{fluoro} was incubated with an equal volume of 0.16 g/ml spheroplasts for 30 min at RT. Spheroplasts
were pelleted to remove unbound excess BtuF$_{\text{fluo}}$. The pellet was washed with resuspension buffer containing 0.25 M sucrose, 0.05 M Tris-HCl pH 8 and 3 mM MgCl$_2$. Spheroplasts with bound BtuF$_{\text{fluo}}$ were resuspended at 0.08 g/ml spheroplasts and dissolved with 1.7 % (w/v) SDS. The fluorescence associated with the sample was measured with a plate reader using excitation at 485 nm and emission at 516 nm. The amount of bound BtuF$_{\text{fluo}}$ was determined by using a BtuF$_{\text{fluo}}$ calibration curve.

4.3.8 Substrate transport assay with spheroplasts

Spheroplasts and BtuF preincubated with nanobodies and cyanocobalamin for 1 hour were equilibrated to RT. 5 µM BtuF, 75 µM nanobodies, 15 µM cyanocobalamin ($^{57}$Co-B12, MP Biomedicals 06B-430000, 10.5 µCi) and 0.08 g/ml spheroplasts (~ 0.45 µM BtuCD) were used for the reaction and the buffer was composed of 0.25 M sucrose, 0.05 M Tris-HCl pH 8 and 3 mM MgCl$_2$. Time points were taken by diluting 50 µl of the reaction in 300 µl ice cold stop buffer composed of reaction buffer supplemented with 8 % (w/v) PEG6000 and 100 µM cold B12. The stopped reaction mix was transferred to a manifold filtration system (Millipore MSFBN6B) and washed twice with 200 µl cold stop buffer. The trapped radioactivity on the filters was measured with a γ counter and initial transport rates were determined by linear regression in Graphpad Prism 7. The transport rates were corrected for BtuCD concentration in the spheroplasts.

4.3.9 Purification and crystallization of the Nb9-BtuF complex

Purified, tagless BtuF was mixed with Nb9 using a 1.3 - fold molar excess of nanobody. The complex was isolated by size-exclusion chromatography using a superdex 200 10/300 GL column equilibrated with 10 mM Tris-HCl pH 7.5 and 100 mM NaCl. The sample was concentrated to approximately 20 mg/ml and 17 % excess nanobody was added to the final sample. Crystallization experiments were performed at 20 °C with home-made and commercial screens using 1:1 and 2:1 drop ratios. Crystals grew in various conditions and initial hits of the best diffracting crystals were obtained in JCSGIV B9 (100 mM Tris-HCl pH 8.5, 200 mM MgCl$_2$, 30 % (w/v) PEG4000) in a 1:1 drop ratio. Transparent bipyramidal crystals appeared after 2 days and grew to full size (about 250 µm) within a few days. For data collection, crystals were cryoprotected with 25 % (v/v) glycerol.
4.3.10 Structure determination of the Nb9-BtuF complex

Data collection was performed at the Swiss Light Source at PSI Villigen. Data processing was done with the XDS package. The structure of Nb9-bound BtuF was determined by molecular replacement in Phenix using apo-BtuF (PDB ID 1N4D) and a nanobody scaffold (cleaved CDRs) as search models. Phasing was not straight forward and a stepwise process using partial solutions from previous runs had to be performed iteratively until all chains in the asymmetric unit were found. Two nanobody chains had to be placed in manually because of poor electron density for automated phasing. Six copies of the Nb9-BtuF complex, equivalent to 12 protein chains in total, were present in the asymmetric unit (Fig. 4.1A). Therefore, strict NCS was used in refinement throughout the structure determination process. Chains D and J displayed the best electron density for BtuF and Nb9, respectively, and thus used for model building. For nanobody chain I, only ~50% of the protein chain was visible in the electron density and represented by high B factors for many residues. Ellipsoidal truncation and anisotropic correction were applied to the data using the diffraction anisotropy server (UCLA-DOE LAB, Fig. 4.1B) [23].

Figure 4.1: Composition of the asymmetric unit and output of the diffraction anisotropy server (UCLA-DOE LAB) [23].

A) Cartoon representation shows BtuF in light blue (chains A-F) and Nb9 in green (chains G-L). Six Nb9-BtuF complexes were present in the asymmetric unit and ordered in a helix-like arrangement when viewed from the side. The following BtuF and Nb9 chains formed the biological assembly: A + H, B + I, C + G, D + J, E + K, F + L. B) Shown are the recommended resolution limits along a*, b*, c* are 2.8 Ang, 3.0 Ang, 2.6 Ang. The recommended resolution limits along a*, b*, c* are 2.8 Ang, 3.0 Ang, 2.6 Ang.
4.4 Results and discussion

4.4.1 Selection of BtuF-specific nanobodies

An alpaca was immunized with B12-bound BtuF and a nanobody-coding DNA library was prepared from lymphocytes isolated from blood. The library was panned against BtuF using phage display, and two rounds of panning were necessary to detect specific enrichment over panning against control solutions. 96 clones of the enriched sub-library were sequenced, which revealed 19 nanobodies with distinct sequences. Five sequences were represented several times and defined as major families. Pull-down assays with immobilized nanobodies and tag-less BtuF identified six BtuF-specific nanobodies (Nb7, Nb9, Nb10, Nb14, Nb15 and Nb17, Fig. 4.2A). Nb7, Nb9 and Nb10 belonged to major families, and Nb14, Nb15 and Nb17 were the only nanobodies with this sequence (Fig. 4.2B).
Figure 4.2: Selection of BtuF-specific nanobodies and sequence alignment. A) SDS-PAGE analysis of pull-down assays. His-tagged nanobodies (Nb) were immobilized on Ni-NTA beads and mixed with a 1.5-fold molar excess of tag-less BtuF (F). Controls were performed by omission of nanobody (no Nb) to detect unspecific BtuF binding and a fraction of unspecifically bound BtuF was observed. Gel lanes correspond to 1 = initial mix, 2 = flow through, 3 = wash and 4 = elution. MW, marker proteins with masses indicated on the left. B) Amino acid sequence alignment of the six nanobodies selected in pull-down assays, and the non-binder Nb12, which was a member of the second-largest family identified in panning. The three CDR regions are labeled. The secondary structure elements of Nb9 are indicated above the alignment. Cysteine positions for disulfide bond formation are numbered in green. Note the additional disulfide bond between C72 (CDR2) and C128 (CDR3) in Nb9, Nb15 and Nb10. The ESPript server 3.0 available online was used to generate the alignment [24].
4.4.2 Effect of purified nanobodies on B12 binding and transport

To assay the effect of the obtained nanobodies on BtuCD-F function, in vitro B12 binding and transport assays were performed in the presence of the nanobodies. Substrate binding was analyzed by measuring B12-induced fluorescence quenching of fluorescently labeled BtuF (BtuF\textsubscript{fluoro}), an assay developed and described previously [6] (Fig. 4.3A). The affinity of the B12-BtuF interaction was determined to be $8.3 \pm 1.7$ nM. A nanobody which did not bind BtuF in the pull-down assay, Nb1, was used as a control and had no effect on the affinity ($8.0 \pm 0.9$ nM). Substantial changes in affinity were determined in the presence of the nanobody binders, ranging from $58 \pm 4$ nM (Nb14) to $1.3 \pm 0.3$ µM (Nb7) (Fig. 4.3B and table 4.1). In the presence of Nb9 and Nb10, the affinity of the B12-BtuF binding interaction could not be determined because it was lower than the detectible range of the assay. This identified Nb9 and Nb10 as the strongest potential inhibitors.

In vitro $^{57}$Co-cyanocobalamin transport was measured with spheroplasts prepared from E. coli cells containing over-expressed wild-type (WT) BtuCD (Fig. 4.3C). A hydrolysis-deficient mutant, BtuCD$_{E159Q}$, was used as a negative control. Similar BtuCD expression levels were measured in spheroplasts with WT BtuCD or BtuCD$_{E159Q}$, as determined by the amount of BtuF\textsubscript{fluoro} that was associated with the spheroplasts (Fig. 4.3D). B12 transport was reduced to $30\%$ in the presence of Nb9, but a 5-fold molar excess of nanobody over B12 was required (Fig. 4.3E). A 2-fold molar excess of Nb9 over B12 resulted in $50\%$ remaining activity (data not shown). Reduction of transport was also detected for Nb10 (to $50\%$) and Nb14 (to $70\%$) compared to the uninhibited rate. Nb7, Nb15 and Nb17, however, hardly affected substrate transport even at high nanobody concentrations.
**Figure 4.3: Effect of nanobodies on BtuCD-F function.** A) Schematic of the substrate binding assay. Fluorescently labeled BtuF (BtuF_fluo) was used to measure B12 binding in the presence of nanobody. B) B12 binding to BtuF_fluo. 5 nM BtuF_fluo, 5 µM Nb and B12 concentrations ranging from 0.06 nM to 3.7 µM were used in the assay. Shown are mean and SEM for n = 4, (n = 2 in case of no Nb and Nb17). Apparent K_D values are indicated in table 4.1. Note that Nb1 is a control nanobody that does not bind BtuF. C) Schematic of the spheroplast-based substrate transport and BtuF_fluo binding assays. 57Co-cyanocobalamin transport into spheroplasts overexpressing WT BtuCD was measured in the presence of Nbs. D) The BtuCD expression level in the spheroplasts was determined by the amount of BtuF_fluo associated with the spheroplasts. Cells transformed with a plasmid containing WT BtuCD but without expression induction (‘WT uninduced’) served as a control. The fluorescence was detected using excitation at 485 nm and emission at 516 nm. E) Cyanocobalamin transport in the presence of Nbs. The following concentrations were used: 5 µM BtuF, 15 µM cyanocobalamin, 75 µM nanobodies and 0.08 g/ml spheroplasts (~ 0.45 µM BtuCD). A hydrolysis-deficient BtuD mutant, E159Q, was used as a negative control. Shown are mean and SEM of the transport rates determined by linear regression using 5 time points.
Table 4.1: Apparent $K_D$ values for cyanocobalamin binding to BtuF$_{fluo}$ in the presence of nanobodies. Note that units are presented either in nanomolar or micromolar and indicated are mean and SEM. Binding curves and details are shown in figure 4.3. The affinities in the presence of Nb9 and Nb10 were so low that they could not be determined (n.d.) due to experimental limitations.

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### 4.4.3 Crystal structure of the Nb9-BtuF complex

To visualize the structural basis of the observed inhibitory effects of Nb9, the crystal structure of the Nb9-BtuF complex was determined. The complex was purified by size exclusion chromatography, and SDS-PAGE analysis confirmed the presence of both components (Fig. 4.4).

**Figure 4.4: Purification and crystallization of Nb9-bound BtuF.** A) Size exclusion chromatography of the Nb9-BtuF complex. Note the shift of the elution peak of the Nb9-BtuF complex compared to the individual proteins. Equivalent amounts of protein (710 µg BtuF, 3.2 mg Nb9) were injected in all experiments. B) SDS-PAGE analysis of isolated Nb9-BtuF complex after gel filtration confirmed the presence of both components. C) Crystals of the Nb9-BtuF complex grown in optimized screens containing 100 mM Tris-Cl pH 8.5, 400 mM MgCl$_2$ and 33 % (w/v) PEG4000.
The best crystals allowed data to be collected beyond 2.9 Å resolution, but the structure of the Nb9-BtuF complex was refined to 2.9 Å resolution (table 4.2). The nanobody was bound to BtuF from the same side where BtuC-binding would occur (Fig. 4.5). Mainly polar and electrostatic interactions were present at the Nb9-BtuF interface. The CDR3 loop was inserted approximately 10 Å into the B12 binding-pocket and several BtuF residues involved in B12 binding (Y50, W66, F162, F168 and W196) were contributing to nanobody binding. Extensive hydrogen bonding was found between the side chains of CDR3 and the backbone of BtuF, and a salt bridge was formed by R123 and E245A in BtuF (Fig. 4.6). A hydrophobic patch and aromatic stacking were observed involving CDR3 residues I124, F125, Y136 and BtuF residues F162, F168 and W196. The side chain residues of CDR1 were engaged in direct and indirect hydrogen bonds with the backbone of BtuF, and D52 formed a salt bridge with K223 in BtuF. In CDR2, N81 was involved in direct or water-mediated hydrogen bonding with the side chains of Y50 and S49.
Figure 4.5: Crystal structure of the Nb9-BtuF complex. A) Ribbon diagram of BtuF in light blue, and Nb9 colored green with CDR1, CDR2 and CDR3 regions colored yellow, magenta and salmon, respectively. The amino- and carboxy-termini are indicated with N and C, respectively. B) View onto the substrate-binding and Nb-binding site. Note the stabilizing disulfide bond in dark blue between the CDR2 (C72) and CDR3 (C128) loops. BtuF is displayed in light blue surface representation.
Figure 4.6: Details of the Nb9-BtuF interface. Illustrated are the interactions of BtuF with CDR1 (A), CDR2 (B), CDR3 N-terminal part (C) and CDR3 C-terminal part (D). BtuF is shown in blue and the CDR regions are colored yellow (CDR1), magenta (CDR2) and salmon (CDR3). Oxygen and nitrogen are shown in red and blue. Water molecules are indicated with red spheres. Shown is the sigma A-weighed 2Fo-Fc electron density map contoured at 1σ.
4.4.4 Comparison of the Nb9-BtuF complex to B12-bound BtuF

Nb9 shared an interface of 1054 Å$^2$ with BtuF, which was larger than that of BtuF with B12, 786 Å$^2$, but smaller than the BtuF-BtuC$_2$ interface in the BtuCD-F complex, which amounts to 2612.5 Å$^2$ (PDBe PISA v1.51 [22/09/2014]). The Nb9-BtuF complex superimposed to B12-bound BtuF (PDB ID 1N4A) with an RMSD of 1.53 over 243 atoms (Fig. 4.7). Comparing the two structures, a 9.9 Å opening was observed between the loops gating the entrance of the substrate-binding pocket in Nb9-bound BtuF. Additional structural differences induced by nanobody binding were also observed in the C-lobe of BtuF.

Figure 4.7: Comparison of Nb9-BtuF complex with B12-bound BtuF. Nanobody-bound BtuF is shown in light blue cartoon representation, with the nanobody in green with colored CDRs (CDR1, yellow; CDR2, magenta; CDR3, salmon). B12-bound BtuF (PDB ID 1N4A) is displayed in red and B12 is shown in stick representation with oxygen in red, nitrogen in blue, and cobalt in brown. The two BtuF chains superimposed with an RMSD of 1.53 Å over 243 atoms. Distances of C$\alpha$ position between corresponding atoms in the loops gating the entrance of the substrate-binding pocket are indicated (1: 5.5 Å, Q67 in Nb9-BtuF and 2: 4.4 Å, N165 in Nb9-BtuF). The C-lobe of BtuF displays more pronounced structural changes than the N-lobe.
The functional inhibition of an ABC importer by targeting the SBP with a nanobody was investigated. Nb9 showed the strongest inhibitory effects in substrate binding and transport assays. Under the experimental conditions used for the transport assay, a high nanobody concentration was necessary to compete with the high affinity interactions of B12-BtuF (8.3 nM) and B12-BtuF with BtuCD (21 nM) [25]. The Nb9-BtuF affinity was presumably in the mid-nanomolar range forming a stable complex on gel filtration. Most nanobody-target affinities were determined by surface plasmon resonance measurements to be in the nanomolar range [26, 27].

The alpaca had been injected with B12-bound BtuF, but Nb-BtuF binding in the pull-down assay did not require bound B12 and all nanobodies reduced the B12-BtuF binding affinity. In addition, the structure of the Nb9-BtuF complex confirmed that B12 must have been displaced from the binding site during the selection of this nanobody. Presumably, BtuF lost its bound B12 to high affinity B12 binders like transcobalamin present in the alpaca blood [28]. The Nb9-BtuF binding mode was reminiscent of the general nanobody binding behavior, recognizing conformational epitopes and clefts of their target [29], or inserting the CDRs into the active site of enzymes and acting as inhibitors [30, 31].

The crystal structure of the Nb9-BtuF complex provided the structural basis of the observed functional effects. Due to steric hindrance, Nb9 binding to BtuF was incompatible with simultaneous binding of B12 or BtuCD. Since the other nanobodies also reduced the B12 binding affinity, they presumably bind BtuF in a similar way. Nb9 might have an overlapping BtuF binding site with Nb15, since they share similarities in their CDRs. All the CDR3 residues of Nb9 involved in BtuF binding were conserved in Nb15, except for F125. This residue was engaged in aromatic stacking in a hydrophobic patch and is replaced by a leucine in Nb15. This substitution retains the hydrophobicity, but the aromatic stacking property is lost. All CDR1 residues involved in BtuF binding were conserved in Nb15. In CDR2, residue N81 of Nb9 was substituted with a histidine in Nb15, maintaining the hydrogen bonding properties. Even though Nb9 and Nb15 probably share the same binding site, the strength of their interaction with BtuF is different. Based on the observed functional effects and the structural insight into Nb9-BtuF binding, the interaction of BtuF with Nb9 is most likely stronger than the one with Nb15.

The nanobody binding site on BtuF and the affinity of the Nb-BtuF interaction are two determinants for the effects on substrate binding and transport. Nb7, Nb15 and Nb17 reduced the B12 binding affinity considerably, but did not interfere with its transport. In the transport
Chapter 4

assays, the Nbs are competing with two interactions, B12 binding to BtuF and BtuCD-F complex formation. The distinct competition of the nanobody with these two interactions explains the different effects on substrate binding and transport observed for some of the nanobodies.

Nanobodies have been used for several purposes other than functional inhibition. They were successfully used as crystallization chaperones because of their ability to rigidify flexible regions, to mediate crystal contacts or to stabilize conformational states of enzymes and transporters [32, 33]. Nanobody-based products like chromobodies (Nb coupled to a fluorescent protein, i.e. GFP) are also helpful diagnostic tools to image the localization of the target protein within the cells or quantify expression [34-36]. In the field of drug discovery, nanobody-drug-conjugates have a potential as clinical therapeutics, because they bind specifically to their target and are less immunogenic than conventional antibodies [37-39]. The herein described approach to inhibit the uptake of essential nutrients by targeting the SBP with a nanobody might have a value for targeting pathogenic bacteria, thus offering a novel antibiotic strategy. This will likely require affinity maturation, which can be performed in a rational way if crystal structures of the nanobody-target complex such as the Nb9-BtuF structure are available [40].
### Table 4.2: Data processing and refinement statistics

Highest-resolution shell values are shown in parentheses and numbers in brackets are after ellipsoidal truncation and anisotropic correction. The correlation coefficient is abbreviated CC [41].

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References


5. Conclusions and Outlook

The uptake of nutrients from the environment is a crucial process for the growth and survival of living cells. The presence of back-up systems to ensure constant supply of certain nutrients is essential. For the uptake of iron in *E. coli* for example several backup systems exist, like the heme and iron-siderophore transport systems [1]. For the uptake of vitamin B12 however, no such back-up system has been described and BtuCD-F is the only vitamin B12 transporter in *E. coli*. BtuCD-F was described to transport vitamin B12, but before the individual *BtuC*, *BtuD* and *BtuF* genes were identified, early *in vivo* data suggested that the B12 uptake system in *E. coli* might also transport the structurally related, but considerably smaller substrate cobinamide. However, *in vitro* cobinamide transport by BtuCD-F was not shown [2].

In this thesis, a liposome-based transport assay was developed to study BtuCD-F catalyzed transport. This method required radiolabeled substrates, whereas radiolabeled B12 was commercially available, the synthesis of radiolabeled cobinamide needed to be established in collaboration with synthetic chemists. The results demonstrated that BtuCD-F could catalyze cobinamide transport *in vitro*. The cobinamide transport rate, which was determined to be 1 -2 nmol cobinamide / mg protein / min, was similar to the one measured for vitamin B12 transport. Cobinamide translocation was also BtuF- and ATP-dependent. The ability of BtuCD-F to translocate cobinamide in addition to vitamin B12 is reminiscent of a back-up system. *E. coli* is not capable of *de novo* cobalamin synthesis, but it can use cobinamide as a precursor and complete the third step of the synthetic pathway, which is the addition of the 5,6-dimethylbenzimidazole (DMB) and phosphoribosyl linker [3]. Therefore, under scarce vitamin B12 conditions, BtuCD-F can take up cobinamide and use it as a source to produce cobalamin.

The substrate-binding protein BtuF bound vitamin B12 and cobinamide with nanomolar affinity, although a 4-fold higher affinity was determined for vitamin B12. The crystal structure of cobinamide-bound BtuF identified a key residue, W66, facilitating the binding of the smaller substrate. A mutagenesis study of this residue showed that high affinity cobinamide binding was dependent on the properties of the residue at this position, while B12 binding was hardly affected by its mutation. This indicated that the binding protein BtuF was more adapted for the specific interaction with cobinamide than with vitamin B12.

A mutagenesis study of the substrate translocation pathway of BtuCD, showed an opposite results for the transmembrane component. Cobinamide transport tolerated more modifications
to the translocation pathway than vitamin B12 transport. Introduction of larger side chains at distinct positions within the substrate translocation pathway abolished vitamin B12 transport, but were tolerated by cobinamide transport. This indicated that the substrate size was screened by the translocation pathway in BtuCD, which thus contributed to the substrate specificity of the import system. Additionally, methionine residues at the entrance of the translocation pathway were crucial for vitamin B12 transport, unlike cobinamide transport, for which mutations to alanine were tolerated. The results of the mutagenesis study of the substrate-binding pocket in BtuF and the translocation pathway in BtuCD, suggested that the BtuCD-F uptake system screens the substrate at two stages, with the substrate-binding protein and in the translocation pathway. BtuF adapted for the facilitated binding of the smaller substrate cobinamide and BtuCD evolved for screening the substrate size. The substrate specificity of ABC import systems mainly lies in the SBP, but these results indicate that the transmembrane component also contributes to substrate specificity.

Nevertheless, the molecular details of the interaction of the two structurally distinct substrates with the translocation pathway remains unknown. What is the orientation of the corrin ring inside the cavity found in the nucleotide-bound state of BtuCD-F? Is vitamin B12 translocated in a base-on (DMB coordinated to the Co atom) or base-off conformation? To address these questions, high-resolution structures of the substrate-bound state are required. Therefore, the resolution of BtuF in the BtuCD-F structures has to be improved and the substrate has to be trapped in the translocation pathway.

I set out to obtain higher resolution structures by using a Fab fragment that bound BtuF and would potentially stabilize its interaction with BtuCD in the crystals. Alternatively, covalent attaching of BtuF to BtuCD was explored to reduce the flexible interaction. The results of both approaches showed that the samples were not compatible with structural studies using X-ray crystallography, but can be potential targets for cryo-electron microscopy. The binding mode of the Fab could be particularly useful for particle alignment of the full transporter, while the sample of covalently attached BtuF to BtuCD showed to be stable at low concentrations typically used in cryo-electron microscopy.

Trapping vitamin B12 or cobinamide within the transporter has proven unsuccessful in detergent micelle, by own efforts and previously published results. The absence of a substrate-binding site in the transporter is the main reason for this difficulty [4]. However, substrate trapping has been described in liposomes, presented in this thesis and also published previously [5]. In this case the trapping is favored by a more rigid environment compared to
the flexible detergent micelle. Reconstitution of membrane proteins into amphipols or nanodiscs could offer similar environments for the proteins and can be used for structural studies using cryo-electron microscopy. In addition, the results showed here demonstrated that histidine residues introduced in the translocation pathway could bind cobinamide, which could help to trap this substrate in the transporter.

The recent advances in the field of cryo-electron microscopy made it possible to obtain high-resolution structures visualizing side chains or bound substrates of transporters similar in size as BtuCD-F [6, 7]. The structure of multidrug resistance protein MRP1 bound to one of its substrate, leukotriene C4, was solved to 3.3 Å resolution and revealed the molecular basis of the interaction of the transporter and the substrate. Cryo-electron microscopy is therefore likely the method of choice to obtain a high resolution representation of BtuCD-F revealing the structural details of the BtuF-BtuC2 interface and interaction of the substrate with the transmembrane component.

Besides BtuCD-F, another ABC type II importer for which transport of distinct substrates has been reported is the Sinorhizobium meliloti homolog of HmuUV, which was shown to transport hemin, ferrichrome and ferrioxamine [8]. However, the molecular interaction of the transporter with the different substrates is unknown. Also the maltose/maltodextrin uptake system MalFGK2-MBP can transport maltose and oligomers until maltoheptaose [9, 10]. Crystal structures of substrate-bound states of MalFGK2-MBP revealed the molecular basis of the interaction of the substrates with the membrane component and explained how larger substrates are accommodated [11]. Therefore structural characterization of the substrate bound state of ABC transporters is necessary to understand the substrate selectivity of the system.

In a final approach, BtuCD-F has proven successful as a model system to study nanobody-mediated inhibition of an ABC importer and provided the framework for a novel antibiotic strategy. ABC importers are exclusively associated with bacteria, which makes them a potential target for drug development due to less cross-reactions with the host [12]. The pathogenesis of bacteria has been linked to the function of ABC importers ensuring nutrient acquisition and thus granting energy for growth. The Yersinia pestis or Staphylococcus aureus heme importer, the Salmonella enterica serotype Typhimurium glutamine importer and the Streptococcus pyogenes or Vibrio cholerae maltodextrin importers could be potential targets for nanobody-mediated inhibition and production of novel antibiotics [13-18].
The mammalian ABC exporter P-glycoprotein (P-gp) and the lipid-linked oligosaccharide flippase PglK are the only other ABC transporters, for which nanobody-mediated inhibition has been reported [19, 20]. The crystal structure of the Nb-P-gp complex revealed the nanobody-binding site at the C-terminal part of the first NBD. Unlike the inhibition of BtuCD-F by Nb9 blocking BtuF, the nanobody bound to P-gp restricted dimerization of the NBDs for ATP hydrolysis. A similar inhibition mechanism was observed for PglK. In addition to the use of nanobodies for functional inhibition, they have also proven successful for structural studies. A conformational selective nanobody was key to stabilize the active state of the G protein-coupled receptor, beta(2)adrenoceptor, by acting as G protein surrogate [21]. The crystal contacts were primarily mediated by the nanobody, which thus also served as crystallization chaperon, a useful tool especially for crystallization of membrane proteins. Nanobodies were also used to improve crystal diffraction and increase resolution, which was crucial to determine the structure of two members of the solute carrier transporter family [22, 23]. Thus, nanobodies served as crystallization chaperones by rigidifying flexible regions, mediating crystal contacts, or stabilizing conformational states [24]. In this regard, the selection of nanobodies compatible with binding to the full BtuCD-F complex could help increasing the resolution of current structures, or stabilize the substrate-bound state of BtuCD-F.

Regarding antibiotic strategies, bacterial import systems could be used as an import pathway for antibiotics, by modifying the natural substrate with a toxin (antibiotic-conjugate). Exemplified with the BtuCD-F uptake system, the smaller substrate cobinamide could be attached to a toxic compound and be used as a delivery system. A similar study has been performed for the SBP FhuD of the hydroxamate-type siderophore transport system related to BtuCD, which could bind and deliver the antibiotic albomycin besides the natural substrates [25]. This shows that ABC import systems can be used as delivery pathways or be the direct target of an antibiotic. Further structural and mechanistic studies including the rational design of substrate-drug-conjugates are necessary to explore ABC import systems for novel antibiotic strategies.

References

Chapter 5

Curriculum Vitae

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Chapter 5

Acknowledgements

I would like to thank my PhD supervisor Prof. Dr. Kaspar Locher for giving me the opportunity to work on this fascinating and challenging project. I am very grateful for his continuous support, guidance and advices throughout the whole thesis and the great training I obtained in his laboratory.

I wish to thank Prof. Dr. Raimund Dutzler and Prof. Dr. Rudolph Glockshuber as the co-examiners of my thesis and reviewing this work. I additionally thank Prof. Dr. Raimund Dutzler for the collaboration regarding the nanobody generation in alpaca.

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I appreciate the support received regarding data collection and initial processing by the beamline staff at PSI Villigen.

I would like to thank all the lab members for their help and a great working experience. A special thanks to Ana Ramirez for her support and friendship.

Last but not least I am grateful to my family and Thomas for their love and support.
Two approaches for structural studies of BtuCD-F in distinct states

Crystallization of membrane proteins is challenging, since a large part of the protein is buried in the detergent micelle and not available for crystal contacts. In BtuCD-F, the crystal contacts involved BtuD and external loops of BtuC [1-3]. BtuF however, was not involved in crystal contacts and it is poorly resolved in the electron density. Therefore, the structural details of the interface between BtuF and BtuC and the changes required for BtuF binding and release are unknown. Two salt bridges formed between BtuF residues E74 and E202 and BtuC residue R56, one from each BtuC subunit, have been shown to mediate BtuF binding [1]. The deletion of analogous residues in the substrate-binding protein of related transporters have been shown to abolish transport due to impaired complex formation [4, 5]. Potential reasons for the poor density of BtuF are the intrinsic flexibility of the BtuF-BtuCD interaction, the two orientations BtuF adopts in the asymmetric state of BtuCD-F and the possible lack of 100 % occupancy. To obtain higher resolution of BtuF in distinct states of the transport cycle, I set out to stabilize the BtuF-BtuC interaction. The first approach was co-crystallization of BtuCD-F with a BtuF-binding Fab fragment. A second approach consisted in stabilizing the interaction of BtuF and BtuC$_2$ by engineered disulfide cross-linking.
Appendix

Appendix I – Antibody fragment targeting of BtuF

A recombinantly expressed and purified Fab fragment from a previously raised antibody against BtuF was tested as potential helper to improve the resolution of BtuF in the BtuCD-F structures. Expression was optimized in HEK 293E cells grown in suspension. Purified Fab was used for binding tests monitoring co-elution on size-exclusion chromatography and BtuF-binding was confirmed including complex formation with BtuCD-F. The bound Fab did not interfere with ATPase or B12 transport activity indicating sound coupling between BtuF and nucleotide binding to BtuCD. The crystal structure of the Fab-BtuF complex revealed the Fab-binding site, which was not optimal for co-crystallization with BtuCD-F, but potentially helpful for structural studies using cryo-electron microscopy. Therefore, BtuCD-F was reconstituted into amphipols with and without Fab and initial negative stain images of BtuCD-F showed a homogenous sample. The lack of available microscope time, however, hindered the progression of the project in this direction.

AI.1 Experimental procedures

AI.1.1 Recombinant expression of Fab

The Fab approach was based on initial work performed by K. Locher, R. Hvorup and V. Korkhov. Immunization of a mouse with liposome-reconstituted BtuCD-F and production of stable hybridoma cells was performed by K. Locher. The selection of a BtuF-binding antibody (Kal0#5), antibody isotyping and the generation of recombinant Fab expression vectors were accomplished by R. Hvorup. Re-cloning into vectors for expression in HEK 293 cells was carried out by V. Korkhov, one vector containing the heavy chain (pUXLH) and a second the light chain (pUXL; Fig. AI.1). The heavy chain construct included a His_{10}-tag for IMAC purification. The vectors were co-transfected and both contained a N-terminal Gaussia luciferase secretion signal. Expression in 293E suspension cells was optimized by varying the DNA to linear PEI ratio and the amount of secreted protein was monitored over several days. Cells were transfected at 0.7 - 0.8 Mio cells/ml in freestyle medium (Thermo Fischer 12338018). The secreted Fab was harvested in 50 ml sterile falcons and the cells were pelleted
by centrifugation (200 g, 5 min, RT). The Fab-containing supernatant was collected and cells were resuspended in fresh medium and transferred back to the original flasks. The supernatant was either used directly for purification or stored at 4°C with 10 mM sodium azide.

**Figure AI.1: Fab expression constructs.**

The Fab genes were inserted into pUXER-based vectors for secreted protein expression in HEK 293 cells. GlucSS; Gaussia luciferase signal sequence (GlucSS).

### AI.1.2 Fab purification

Small-scale batch purification from expression tests were performed with 25 ml supernatant. The supernatant was supplemented with 10 mM Tris-HCl pH 7.5, 20 mM imidazole-HCl pH 8 and 0.5x PBS pH 7.3. The Fab fragment was bound to 100 µl Ni-NTA beads overnight at 4°C. The mix was loaded on a 5 ml mini-column (Sigma-Aldrich C2728-200EA) and the resin was washed with 20 column volumes (CV) of 1xPBS supplemented with 20 mM imidazole-HCl pH 8. The protein was eluted with 3 CV of 1xPBS containing 300 mM imidazole-HCl pH 8. 20 µl of the eluent was loaded on 15 % reducing SDS-PAGE for analysis.

For large-scale purification, the supernatant was subjected to a second spin after harvesting (16k rpm, SA-600, 30 min, 4°C) to remove remaining cell debris and supplemented with 10...
mM Tris-HCl pH 7.5, 20 mM imidazole-HCl pH 8 and 0.5x PBS pH 7.3. The sample was loaded on a XK 16 column containing 3 ml Ni-NTA resin equilibrated with 50 mM Tris-HCl pH 7.5, 200 mM NaCl and 25 mM imidazole-HCl pH 8. The resin was washed with 30 CV of the same buffer and followed by 15 CV of a second wash with 50 mM imidazole-HCl pH 8. The bound Fab was eluted with 300 mM imidazole-HCl pH 8 and desalted into 50 mM Tris-HCl pH 7.5, 200 mM NaCl and 0.5 mM EDTA-NaOH pH 8. The stability of the sample was analyzed by size-exclusion chromatography (SEC) using a superdex 200 10/300 GL column equilibrated with desalting buffer. All steps were performed at 4 °C.

**AI.1.3 Binding assay of Fab to BtuF**

Fab binding to BtuF was assayed on gel filtration using a superdex 200 10/300 GL column equilibrated with 10 mM Tris-HCl pH 7.5 and 100 mM NaCl. 10µM (0.5 mg/ml) Fab was mixed with 20 µM (0.57 mg/ml) BtuF6H and incubated for 15 min on ice. Binding was assayed in the absence and presence of 1 mM cobinamide (Cbi). 500 µl sample was injected and protein and Cbi elution were monitored at 280 nm and at 360 nm, respectively.

**AI.1.4 Reconstitution of Fab-bound BtuCD-F into amphipols**

BtuCD-F was purified as described in chapter 3 but changing the detergent from 0.01 % (v/v) C\textsubscript{12}E\textsubscript{8} to 0.016% DDM. Reconstitution of detergent-purified BtuCD-F into amphipol was performed according to a previously described protocol [6, 7]. The protein was mixed with amphipols (A8-35) using a ratio of 1:4 or 1:5 (w/w) BtuCD-F to amphipols and incubated rotating for 4 hours at 4°C. Detergent was removed with two bio-bead steps at 4°C (BioBeads SM-2, BioRad 152-8920), the first one overnight and the second one for 30 min. The sample was purified by SEC using a superdex 200 10/300 GL column equilibrated with 20 mM HEPES pH 7 and 150 mM NaCl.

**AI.1.5 ATPase and substrate transport assays**

The procedures for ATPase and substrate transport assays using spheroplasts are described in chapter 3 and 4. Details of the concentrations used in the assays are indicated in the figure legends.
**AI.1.6 Crystallization of the Fab-BtuF complex**

Purified Fab and BtuF were mixed using a 2-fold molar excess of BtuF over Fab and supplemented with 0.2 mM B12. The sample was incubated for 30 min on ice and concentrated (30 kDa cutoff) to a final volume of 500 µl for injection on a superdex 200 10/300 GL column equilibrated with 10 mM Tris-HCl pH 7.5 and 100 mM NaCl. The purified Fab-BtuF complex was concentrated to 14 mg/ml and supplemented with 0.6 mM B12. Three commercial screens (Hampton Index, Crystal and PEG/ION) and a home-made initial screen were set up using two protein concentrations (14 mg/ml and 8 mg/ml). Plates were set up at 1:1 and 2:1 drop ratios and incubated at 20°C. Crystals were cryoprotected with 25 % (v/v) glycerol for diffraction experiments.

**AI.1.7 Data collection and structure determination**

Crystallographic data was collected at the Swiss Light Source (SLS) at the Paul Scherrer Institut in Villigen. Diffraction images were collected on a pilatus detector 6M. The structure of Fab-BtuF complex was solved by molecular replacement using BtuF (PDB ID 1N2Z) devoid of B12 and a Fab with 75 % sequence identity (PDB ID 1SFI) as search models.

**AI.2 Results and discussion**

**AI.2.1 Recombinant Fab expression and purification**

Fab expression was optimized in HEK 293E suspension cultures using co-transfection of the heavy and light chain vectors. Initial expression tests were performed in 25 ml cultures varying the concentrations and ratios of linear PEI and total DNA, which contained equal amounts of heavy and light chain DNA. The supernatant was harvested after 2, 4, 6 and 8 days after transfection and subjected to batch purification for Fab expression analysis. All the tested conditions showed Fab expression and the combination of 4 µg/ml PEI and 0.5 -1 µg/ml total DNA had the highest yields (Fig. AI.2). Fab expression increased over time, with highest expression levels after 6 days. The estimated yield over 8 days was about 2 mg Fab / l culture.
**Appendix**

### Figure AI.2: Small-scale Fab expression texts.

A) HEK 293E suspension cells were transfected at a density of 0.8 Mio cells/ml in 25 ml freestyle medium in 250 ml flasks and different linear PEI and DNA ratios were tested. The supernatant was harvested after 2, 4, 6 and 8 days post transfection (p.t.). Each condition was analyzed in three independently transfected and grown cultures. Two conditions could not be analyzed throughout the experiment in triplicates due to contamination (empty lanes). HC, heavy chain; LC, light chain. B) Shown is the estimated amount of Fab in the supernatant after 2, 4, 6 and 8 days post transfection (p.t.). The color-coding corresponds to the one in panel A. C) The samples of the first column in panel A (yellow star) were loading on the same gel for direct comparison.

For large-scale expression, 4 µg/ml linear PEI and 0.5 µg/ml total DNA were used. The volume of the cultures could be scaled-up to 300 ml maintaining the yield obtained for small-scale expressions. Increasing to 600 ml cultures reduced the expression yield of Fab.
(Fig. AI.3), presumably because of the limitation of the 2 l flask used for growing the culture.

**Figure AI.3: Large-scale Fab expression.** The expression in different culture volumes was tested in parallel. Gel lanes correspond to 1, 600 ml; 2, 300 ml; 3, 100 ml; 4, 50 ml; 5, 25 ml. The HEK 293E suspension cells were transfected at a density of 0.7 Mio cells/ml using 4 µg/ml linear PEI and 0.5 µg/ml DNA. The supernatant was harvested after 5, 9 and 13 days post transfection (p.t.). Initial test purifications were performed with 25 ml of each culture and 10 µl elution was loaded on 15 % reducing SDS-PAGE for expression analysis.

**Fab purification**

Large-scale purifications showed pure Fab preparation with a yield of about 4 mg Fab/ml culture. Purified Fab was analyzed by SEC, showing a stable and monodisperse peak (Fig. AI.4). The purity of the sample was analyzed by SDS-PAGE and the presence of the heavy and light chain was confirmed.

**Figure AI.4: Fab purification.** A) SEC analysis of the purified Fab using a superdex 200 10/300 GL column equilibrated with 50 mM Tris-HCl pH 7.5, 200 mM NaCl and 0.5 mM EDTA-NaOH pH 8. The protein concentration was 0.25 mg/ml and 200 µl were injected. B) Reducing 15 % SDS-PAGE analysis of the purified sample. Gel lanes correspond to 1, supernatant; 2, flow through; 3, wash 1; 4, wash 2; 5, elution; 6, pellet.
Complex formation of Fab with BtuF and BtuCD-F

Binding of the recombinantly expressed Fab to BtuF was analyzed using SEC and monitoring co-elution of the two proteins. A clear shift of the elution peak towards larger hydrodynamic radius was observed for the Fab-BtuF complex compared to the individual proteins (Fig. AI.5). The presence of Fab and BtuF in the peak fraction was confirmed by SDS-PAGE analysis. The difference in size allowed isolation of the complex for further crystallization experiments. The presence of the BtuF substrate cobinamide did not interfere with Fab binding.

**Figure AI.5: Fab-BtuF binding tests.** **A-B)** Purified proteins were mixed using 10 μM Fab (0.5 mg/ml) and 20 μM BtuF (0.57 mg/ml) and incubated for 15 min prior to injection of 500 μl. In panel B, 1 mM cobinamide (Cbi) was added for another 15 min before injection. The SEC experiment was performed with a superdex 200 10/300 GL column equilibrated with 10 mM Tris-HCl pH 7.5 and 100 mM NaCl. Cbi elution was monitored at 360 nm. **C)** 15 % non-reducing SDS-PAGE analysis. The fractions corresponding to Fab-bound BtuF in panel A (peak no 1) and B (peak no 2) confirmed the presence of Fab and BtuF. Lane 1, peak 1; lane 2, peak 2. **D)** The Fab-BtuF complex showed a clear shift of the elution peak compared to the individual proteins. The blue trace is the same as in panel A.
Binding of Fab to the BtuCD-F complex was analyzed by SEC and SDS-PAGE (Fig. AI.6). A clear shift towards larger hydrodynamic radius was not observed for Fab-bound BtuCD-F compared to BtuCD-F. However, a 1.2-fold molar excess of BtuCD-F was used over Fab and no unbound Fab was detected, indicating that all Fab was co-eluting with BtuCD-F.

Figure AI.6: Fab-BtuCD-F binding test in detergent. A) Purified proteins were mixed using a 1.2-fold molar excess of BtuCD-F over Fab. The SEC experiment was performed with a superdex 200 10/300 GL column equilibrated with 10 mM Tris-HCl pH 8, 100 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 0.01 % C12E8. B) 15 % non-reducing SDS-PAGE analysis. Lane number 1, 2 and 3 correspond to peak 1, 2 and 3 in panel A.

AI.2.3 Crystal structure of the Fab-BtuF complex

For crystallization experiments, purified BtuF and Fab were mixed and the complex was isolated by SEC. The sample was supplemented with 0.6 mM B12 prior to the crystallization experiments. Red crystals grew to a size of approximately 300 µm and diffracted beyond 2.6 Å resolution (Fig. AI.7). The crystal structure of the Fab-BtuF complex was determined to 2.3 Å resolution using molecular replacement (Fig. AI.8 and table AI.1). The structure revealed the Fab-binding site on BtuF and the molecular details of the interaction interface. The Fab was bound to the N-lobe of BtuF and B12 was present in the substrate-binding pocket of BtuF. This confirmed the observation made in the BtuF binding test that Fab does not interfere with substrate binding. Mainly polar interactions were present at the binding
interface. Alignment of Fab-bound BtuF to BtuCD-F revealed how the Fab was sticking out (Fig. AI.9).

Figure AI.7: Fab-BtuF crystals and diffraction pattern. A) Crystallization experiments were performed with 14 mg/ml Fab-bound BtuF supplemented with 0.6 mM B12. Crystals were obtained in 50 mM HEPES sodium pH 7.0, 1 % (w/v) tryptone and 20 % (w/v) PEG3350. B) Representative diffraction pattern. Data collection was performed using filter 0.5 transmission, 0.5 s exposure time, 0.5° angle increment and a wavelength of 1 Å. Resolution rings are 6.86 Å, 3.51 Å and 2.43 Å.

Figure AI.8: Crystal structure of the Fab-BtuF complex. Protein chains are shown in cartoon representation and the amino- and carboxy-termini of BtuF are indicated. BtuF residue stretch 221 to 229 was not visible.
AI.2.4 Preparation of Fab-bound BtuCD-F in amphipols

The structure of the Fab-BtuF complex revealed the binding site of the Fab, which was expected to be not optimal for co-crystallization with BtuCD-F. It would presumably lead to a huge solvent content of the crystals and potentially interfere with the crystal packing observed in previous BtuCD-F structures. For cryo-electron microscopy studies however, a binding partner sticking out like the Fab is very helpful for particle alignment. Additionally, the unchanged functional activity of BtuCD-F in the presence of the Fab (data shown in next section) indicated that the Fab did not impose changes to the transporter, which made it a suitable binder for structural studies.

The structure of a mammalian TRP channel was solved to 3.4 Å resolution using cryo-electron microscopy, thus breaking the side-chain barrier for membrane proteins with a technique other than X-ray crystallography [7]. One of the crucial steps was an optimal sample preparation achieved by reconstitution of the sample into amphipols. Additionally, recent advances in cryo-electron microscopy have lead to the structural characterization of substrate recognition by a mammalian exporter MRP1 or mechanistic insight into the ABC transporter related ion channel CFTR [9, 10]. Therefore, high resolution crystal structures of
distinct states of BtuCD-F could be obtained using cryo-electron microscopy and provide structural insight into substrate recognition or the BtuF-BtuC₂ interface.

In analogy to the sample preparation of the TRP channel, Fab-bound BtuCD-F was reconstituted into amphipols. The reconstitution was performed with two ratios of BtuCD-F to amphipol and both yielded stable preparation judged by the monodisperse peak analyzed by SEC (Fig. AI.10). SDS-PAGE analysis confirmed the presence of all four components Fab, BtuF, BtuC and BtuD. Preliminary negative stain results of BtuCD-F without Fab showed a homogeneous sample distribution with some aggregates in the undiluted sample (Fig. AI.11). This was a promising initial result and could be followed up with Fab-bound BtuCD-F.

Figure AI.10: Reconstitution of Fab-bound BtuCD-F into amphipols. BtuCD-F was mixed with amphipols in a 1:4 (A) or 1:5 (C) weight-to-weight ratio. A superdex 200 10/300 GL column was equilibrated with 20 mM HEPES pH 7 and 150 mM NaCl and eluted fractions were analyzed by 15 % non-reducing SDS-PAGE. A-B) 300 µl of a 1 mg/ml BtuCD-F solution was injected (blue trace). A 1 molar excess of BtuF was added to BtuCD-F to ensure full BtuF occupancy of the transporter (green trace). A 1.2 - fold molar excess of Fab over total BtuF was added for preparation of a Fab-bound BtuCD-F complex (black trace). BtuF alone was injected as control (red). Note the shift in elution volume of the BtuCD-F-Fab complex compared to BtuCD-F alone. C-D) The same ratios were used to prepare the BtuCD-F-Fab complex as described for panel A-B.
Figure AI.11: Negative stain electron micrographs of BtuCD-F in amphipols. Sample concentrations were 0.4 mg/ml BtuCD-F and a 10-fold dilution. Buffer composition was 20 mM HEPES pH 7.2 and 100 mM NaCl. Electron microscopy work performed by the Raunser lab.

**AI.2.5 Functional activity of Fab-bound BtuCD-F**

The effect of the Fab on the functional activity of BtuCD-F was measured by ATPase activity and substrate transport assays (Fig. AI.12). The ATPase activity of BtuCD-F in 0.016 % v/ DDM was determined to be 187 ± 3 nmol P / mg protein / min, which was not altered by the presence of the Fab (192 ± 4 nmol P / mg protein / min). A similar result was obtained for the ATPase activity of BtuCD-F reconstituted into amphipols, which was around 40 nmol P / mg protein / min and not affected by the presence of the Fab. The ATPase rate measured in amphipols was about 4-fold slower than the one in detergent, indicating an altered dynamics of the BtuCD-F complex in the more rigid amphipol belt.

*In vitro* cyanocobalamin transport was measured using spheroplasts containing over-expressed wild-type BtuCD (Fig. AI.12C). A hydrolysis-deficient mutant of BtuD, E159Q, was used as a negative control. Similar BtuCD expression levels were determined for the two spheroplast preparations. The binding of the Fab fragment did not affect substrate transport.
Figure AI.12: Functional activity of Fab-bound BtuCD-F. A) ATPase activity in detergent. 0.02 mg/ml BtuCD-F (0.13 µM) and a 2-fold molar excess of Fab over CDF (0.26 µM) were used. ATPase activity was measured at RT using 1 mM ATP and 5 mM MgCl$_2$ in 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 0.016 % v/v DDM. Shown are mean and SEM of n = 3. B) ATPase activity in amphipols. 1:4 and 1:5 indicate two separate preparations of BtuCD-F in amphipols (see Fig. AI.10). The ATPase activity was measured under the same condition as described in panel A with 0.05 mg/ml protein in 20 mM HEPES pH 7 and 150 mM NaCl. C) Schematic of the transport assay and determination of the BtuCD concentration in spheroplasts using a fluorescently labeled BtuF (BtuF$\text{fluor}$). D) BtuCD expression level in spheroplast. Spheroplasts were incubated with BtuF$\text{fluor}$ and the amount of associated BtuF$\text{fluor}$ was used to determine the BtuCD concentration. Cells transformed with a plasmid containing WT BtuCD without expression induction was used as control. E) Cbl transport activity. The transport reaction was measured at RT using 5 µM BtuF, 6 µM Fab, 15 µM Cbl and 0.08 g spheroplast/ml (0.4 µM BtuCD) in 0.25 M sucrose, 0.05 M Tris-HCl pH 8 and 3 mM MgCl$_2$.
**Appendix**

**Table AI.1: Data processing and refinement statistics.** Highest-resolution shell values are shown in parentheses. The correlation coefficient is abbreviated CC [8].

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**Fab-B12-BtuF**
Appendix II – Disulfide cross-linking of BtuF to BtuCD

Disulfide cross-linking has been used for structural and functional studies of membrane proteins [11-13]. This strategy was applied to BtuCD-F to obtain the structure of the nucleotide bound state. An engineered disulfide bond between the two BtuD subunits was crucial to stabilize the closed sandwich dimer formed upon nucleotide binding [3]. To reduce the flexibility of the BtuF-BtuC₄ interaction and ensure 100% occupancy of BtuF on the transporter, disulfide cross-linking was used to covalently attach BtuF to BtuC. The engineered disulfide bond did not change the ATP hydrolysis activity, indicating that the coupling between BtuF and nucleotide binding and hydrolysis in BtuCD was not affected. Initial transport results showed that cross-linking of BtuF to BtuCD displayed an only 2-fold higher transport activity compared to the omission of ATP. Release of the attached BtuF with reducing agent and addition of excess BtuF increased the transport activity 6-fold. This suggested that increasing the local concentration of BtuF by covalent attachment to the transporter did not result in higher transport rate. The presence of an excess of BtuF over BtuCD, which can bind and unbind the transporter, is necessary for efficient transport. The usage of the covalent cross-link of BtuF to BtuCD for crystallization purposes, however, was problematic because of sample aggregation at higher concentrations. Electron microscopy would be an alternative method for structural studies, since this method does not require high sample concentration.

AII.1 Experimental procedures

AII.1.1 Design and cloning of BtuF and BtuC cysteine mutants

To covalently attach BtuF to BtuCD with disulfide cross-linking, four cysteine mutants were introduced at distinct positions in BtuF and BtuC, generating four cross-linking pairs X1, X2, X3 and X4 (Fig. AII.1). Cysteine mutations were introduced into a cys-less BtuCD construct with E159Q and N162C mutations in BtuD. These mutations were necessary to stabilize the transporter in the nucleotide-bound state [3]. E159Q resulted in a hydrolysis-deficient mutant and N162C was used for the disulfide cross-linking to stabilize the NBD dimer. The most efficient BtuF-BtuC cross-link (X4) was re-cloned with wild-type BtuD to enable functional
characterization. The initial BtuF constructs contained a non-cleavable His-tag and two were re-cloned with a cleavable His-tag for optimized purification procedures. The mutations were introduced by site-directed mutagenesis and confirmed by DNA sequencing (Microsynth).

**Figure AII.1: Cross-linking design.** Shown are the positions of the four cross-linking pairs between BtuF (black) and BtuC2 (grey). Three pairs are positioned in the N-lobe of BtuF and one in the C-lobe. The cross-linking pairs are numbered 1 – 4 (X1, X2, X3, X4) and the involved residues are shown in the table. Numbers next to the name of the cross-linking pair indicated the distance between the Cα atoms in Å. The AMPPNP-bound BtuCD-F model (PDB ID 4FI3) was used to design the cross-linking pairs.

<table>
<thead>
<tr>
<th>Cross-link</th>
<th>BtuF</th>
<th>BtuC</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1 (9)</td>
<td>S99C</td>
<td>D187C</td>
</tr>
<tr>
<td>X2 (7.7)</td>
<td>E91C</td>
<td>Q174C</td>
</tr>
<tr>
<td>X3 (7.2)</td>
<td>Q226C</td>
<td>N114C</td>
</tr>
<tr>
<td>X4 (6.3)</td>
<td>Q67C</td>
<td>E302C</td>
</tr>
</tbody>
</table>

**AII.1.2 Purification of BtuF-BtuC cross-linking mutants**

BtuF and BtuC were purified as described in chapter 2, but kept under reducing conditions with 5 mM β-mercaptoethanol until elution to avoid oxidation of the cysteines. The corresponding BtuF and BtuC pairs were purified simultaneously. The purified proteins were desalted into non-reducing conditions and used directly for disulfide cross-linking. Initial cross-linking tests were performed with different BtuF to BtuCD ratios and 0.5 – 1 mM CuCl2 was used to catalyze disulfide bond formation. The cross-linking reaction was performed at RT for 1 hour and stopped with 2 mM N-ethylmaleimide. Cross-linking efficiency was analyzed by non-reducing 17 % SDS-PAGE.

Preparation of cross-linked BtuCD-F for structural and functional studies was performed with a 3-fold molar excess of BtuF. The reaction was stopped with 10 mM EDTA-NaOH pH 8
followed by desalting of the sample into 50 mM Tris-HCl pH 7.5, 500 mM NaCl and 0.1 % w/v LDAO. An optimized procedure required re-loading of cross-linked BtuCD-F on Ni-NTA to remove excess BtuF and simultaneous buffer exchange to 10 mM Tris-HCl, 100 mM NaCl and 0.01 % C_{12}E_8. Cross-linked BtuCD-F was eluted with 200 mM imidazole-HCl pH 8 and purified by SEC with a superdex 200 10/300 GL column equilibrated with 10 mM Tris-HCl, 100 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 0.01 % C_{12}E_8. The sample was analyzed by non-reducing SDS-PAGE and MALDI MS positive mode (in collaboration with the Zenobi laboratory, Fan Chen).

AII.1.3 ATPase activity and substrate transport assays

ATPase activity and substrate transport assays were performed as described in chapter 3. To measure the activity in reducing conditions, 10 mM DTT was added to the mixture, followed by incubation for at least 30 min at RT prior to reaction start. The transport reaction was started by the addition of substrate to the proteoliposomes. In case of additional non-cross-linked BtuF (addBtuF), B12 and addBtuF were pre-incubated and added simultaneously.

AII.2 Results and discussion

AII.2.1 Purification of cysteine mutants and cross-linking tests

The BtuCD cysteine mutants were expressed and purified with similar yields as wild-type BtuCD. Size-exclusion chromatography (SEC) analysis showed a monodisperse peak for all of them (Fig AII.2A). The BtuF cysteine mutants were expressed and purified with 25 – 50 % of the yields obtained for the wild-type. SEC analysis showed a main monodispersed peak and a second smaller peak at earlier elution volume, presumably oligomerized BtuF (Fig AII.2B).
Figure AII.2: Purification of BtuF and BtuCD cysteine mutants. Shown are representative SEC profiles using a superdex 200 10/300 GL column. A) 200 µl of BtuC<sub>Q174C</sub>BtuD<sub>E0NC</sub> (X2) at 0.4 mg/ml was injected and the buffer contained 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 0.1 % w/v LDAO. B) 200 µl of BtuF<sub>6H</sub>E91C at 1.3 mg/ml was injected and the buffer contained 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 10 % (v/v) glycerol.

The BtuF and BtuCD cross-linking pairs were purified simultaneously allowing immediate cross-linking after purification. Cross-linking tests were performed in solution with different BtuF to BtuCD ratios. Attempts to cross-link the pairs X1 and X2 were not successful, resulting in none or only a small fraction of cross-linked BtuF-BtuC based on SDS-PAGE analysis (Fig. AII.3A-B). Mixing the two proteins together under reducing conditions and repetition of the cross-linking reaction on column did not improve the cross-linking of these two pairs (data not shown). Cross-linking of the X3 and X4 pairs was successful, but BtuF also cross-linked with itself (Fig. AII.3C-D). BtuF self cross-linking was observed for all four pairs and was clearly visible by the increase of its dimer band (BtuF:BtuF) when using excess BtuF. The oligomerized BtuF observed in the SEC profile was likely cross-linked BtuF, since the SEC analysis was performed in the absence of reducing agent (Fig. AII.2B). Wild-type
BtuF dimerization has been observed before in crystal packing or in SEC analysis with buffers containing 0.1 % w/v LDAO. Nevertheless, BtuF readily cross-linked with BtuC and only when excess BtuF or no BtuCD was available, it started to cross-link with itself.

Figure AII.3: Cross-linking tests of BtuF to BtuCD. Shown is a non-reducing SDS-PAGE analysis (17%) of the four cross-linking pairs X1 (A), X2 (B), X3 (C) and X4 (D). Monomeric and dimeric BtuF, BtuC and BtuD are indicated by F, C, D or F:F, C:F or D:D. Cross-linking was performed with different BtuF to BtuCD ratios indicated by the gel lane numbers 1 (0.5:1), 2 (1:1), 3 (3:1), 4 (0:1), 5 (1:0) and 6 (10:1). Cross-linking was performed with 0.5 – 1 mM CuCl₂ for 1 hour at RT and stopped by the addition of 2 mM N-ethylmaleimide. Not identified bands are indicated with an arrow.

AII.2.2 Sample preparation of cross-linked BtuCD-F (X4)

Purification of the individual proteins was performed as described above and cross-linked in solution using a 3-fold molar excess of BtuF over BtuCD. Preparative SEC was initially chosen to separate the cross-linked BtuCD-F from excess BtuF. However, no elution of
excess BtuF was detected and SDS-PAGE analysis of the peak corresponding to cross-linked BtuCD-F showed the presence of excess BtuF (Fig. AII.4). This was remarkable, since excess cross-linked BtuF-BtuF (56.7 kDa) should not co-elute with BtuCD-F (157.6 kDa). However, the presence of excess BtuF, cross-linked and not cross-linked, was confirmed by mass spectrometry. The formation of oligomerized cross-linked BtuF-BtuF mixed with monomers was a plausible explanation for this observation. As mentioned before, wild-type BtuF has already been observed to form dimers in buffers containing 0.1 % w/v LDAO. Therefore, SEC could not be used to isolate cross-linked BtuCD-F from excess BtuF.

**Figure AII.4: Analysis of X4 sample preparation.** A) 500 µl of 1 mg/ml BtuCD-F was injected on a superdex 200 10/300 GL column equilibrated with 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 0.1 % (w/v) LDAO. The EDTA-Cu peak comes from cross-linking with CuCl₂ and stopping the reaction with 10 mM EDTA-NaOH pH 8. B) SDS-PAGE analysis. Gel lanes correspond to 1 = BtuF, 2 = BtuCD, 3 = cross-linked BtuCD-F before SEC, 4 = cross-linked BtuCD-F after SEC, 5 – 8 = same as 1 – 4 with reducing loading buffer. C) Mass spectrometry analysis of X4. For the MALDI MS positive mode experiment, the sample was concentrated to 28 µM and for reducing conditions, 24 mM DTT was added.
To optimize the sample preparation of the cross-linking pairs X3 and X4, the BtuF cysteine mutations were introduced into a construct with cleavable His-tag and tag-less BtuF was used for cross-linking. After cross-linking to BtuCD, the sample was reloaded on Ni-NTA to remove excess BtuF in combination with a detergent exchange to 0.01 % v/v C_{12}E_{8}. The procedure was successful according to SDS-PAGE analysis and mass spectrometry confirmed the absence of excess BtuF (Fig. AII.5).

Figure AII.5: Analysis of optimized X4 sample preparation. A) SDS-PAGE analysis. Gel lanes correspond to 1 = BtuF with His-tag, 2 = BtuF with cleaved His-tag, 3 = BtuCD, 4 = BtuCD-F after cross-linking, 5 = desalted cross-linked BtuCD-F before reloading on Ni-NTA, 6 = excess BtuF in Ni-NTA flow through, 7 = cross-linked BtuCD-F after Ni-NTA and SEC purification, 8 = same as lane 4 in figure AII.4B shown for comparison. B) Mass spectrometry analysis of X4. For the MALDI MS positive mode experiment, the sample was concentrated to about 28 µM and for reducing conditions, 24 mM DTT was added.
AII.2.3 Preparation and functional analysis of cross-linked BtuCD-F with wild-type BtuD (X4WT)

For functional analysis, the BtuC mutation E302C of X4 was re-cloned with wild-type BtuD (BtuC<sub>E302C-D<sub>WT</sub></sub>). For simplification, the cross-linked complex BtuC<sub>E302C-D<sub>WT</sub></sub>-BtuF<sub>3C-Q67C</sub> is called X4wt. The optimized preparation procedure established for X4 was applied to X4wt and yielded a stable and efficiently cross-linked sample (Fig. AII.6A-B). For the functional analysis, the condition for reducing the cross-link had to be established in order to compare it to the cross-linked version. The BtuF-BtuC cross-link was not as readily reduced as the BtuD-BtuD cross-link, since the reducing conditions previously used to break the BtuD-BtuD cross-link did not reduce the BtuF-BtuC cross-link (Fig. AII.6C). Incubation with 10 mM DTT for 30 min at RT was necessary to break the BtuF-BtuC cross-link and was used for the functional analysis.

Figure AII.6: Analysis of X4wt sample preparation for functional assays. A) SEC profile of X4wt at 8 mg/ml, 500µl injected. B) SDS-PAGE analysis of X4wt sample preparation. Final sample marked with the red box was used for reconstitution into liposomes. Gel lanes correspond to 1 = tag-less BtuF, 2 = BtuCD, 3 = cross-linked BtuCD-F before Ni-NTA, 4 = excess BtuF in Ni-NTA flow through, 5 = cross-linked BtuCD-F. C) SDS-PAGE reducing analysis of cross-linked X4. The sample was treated with 1 mM DTT for 35 min on ice as previously used to reduce the BtuD-BtuD cross-link. Gel lanes correspond to 1 = cross-linked X4, 2 = cross-linked X4 after treatment. D) Reducing analysis of cross-linked X4wt. Cross-linked sample was incubated with 1 mM (1) or 10 mM DTT (10) for 10 min (red) of 30 min (green) at RT. The reducing reaction was stopped by inactivating DTT with 20 mM N-ethylmaleimide. Numbers next to the gels indicate the molecular weight in kDa.
ATPase and substrate transport assays were performed with X4wt to assay the functional effect of the BtuF-BtuC cross-link. The ATPase activity was not affected by the covalent attachment of BtuF to BtuCD indicating sound coupling of BtuF and nucleotide binding within the transporter. The ATPase rate of X4wt was determined to be $303 \pm 10 \text{ nmol P / mg protein / min}$ and $317 \pm 16 \text{ nmol P / mg protein / min}$ under reducing conditions. The value determined for wild-type non-crosslinked BtuCD-F was $385 \pm 30 \text{ nmol P / mg protein / min}$ (Fig. AII.7).

**Figure AII.7: ATP hydrolysis by X4wt.**

ATPase rate of X4wt in detergent compared to wild-type BtuCD-F. The hydrolysis activity was measured at RT using 1 mM ATP, 5 mM MgCl$_2$, ± 10 mM DTT in a buffer containing 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5 mM EDTA-NaOH pH 8 and 0.01 % (v/v) C$_{12}$E$_8$.v

Preliminary B12 transport was measured *in vitro* using cross-linked BtuCD-F reconstituted into liposomes. ATP was incorporated into the liposome lumen together with an ATP-regenerating system to keep a constant ATP concentration within the time-course of the experiment. Two different substrate concentrations were used and the transport reaction was started by adding B12 alone or B12 bound to 1 µM additional BtuF. Minimal B12 transport was detected for the cross-linked BtuCD-F, since the signal was only 2-fold higher in the presence of luminal ATP compared to its omission (Fig. AII.8). Cross-linked BtuCD-F did not transport faster than when the cross-link was reduced with DTT, indicating that increasing the local concentration by covalent attachment of BtuF did not result in higher transport rate. Supplementation with additional BtuF ($\text{addBtuF}$) to the cross-linked BtuF-BtuC resulted in no detectable activity, because most of B12 was bound to $\text{addBtuF}$ (Fig. AII.8A). Under reducing conditions, however, high transport rates were measured for supplementation with $\text{addBtuF}$. Since the cross-link was broken, $\text{addBtuF}$ could also feed the transporter with B12. This indicated that an excess of BtuF over transporter resulted in an increased transport rate. This condition is similar to the *in vivo* situation, where an excess of periplasmic BtuF is present.
Appendix

compared to BtuCD in the membrane. The transport results also indicated that detachment of BtuF is required for efficient transport (Fig. AII.8B). Therefore, the presence of an excess of B12-loaded BtuF ready to feed the transporter is crucial for efficient transport. Nevertheless, cross-linking BtuF to BtuCD did not fully abolish B12 transport, reminiscent of the transport activity of substrate binding domains fused to the transporter [14, 15]. An interpretation of the results of the transport assay is illustrated (Fig. AII.9)

The signal in the absence of ATP was presumably trapped but not transported B12 (Fig. AII.8A, red arrow). Trapped B12 was not detected in the presence of excess _addBtuF_ indicating a reversible binding and unbinding of B12 to the cross-linked transporter in the absence of ATP. B12 trapping has been detected in liposomes before, but only in the presence of nucleotide [3]. The trapped signal was from B12 bound to the translocation pathway, which formed a substrate cavity when nucleotide was present. The trapped signal observed for the cross-linked BtuF to BtuCD is presumably from B12 bound to the covalently attached BtuF before it forms a complex with BtuCD and the periplasmic BtuC loops intrude into the substrate-binding pocket of BtuF. Attempts to isolate the cross-linked BtuCD-F with trapped substrate in detergent or amphipols were not successful judged by monitoring co-elution of protein and substrate on SEC (data not shown).

![Figure AII.8: Cyanocobalamin transport by X4wt in liposomes.](image)

The transport reaction contained 4 mg/ml liposomes (0.3 µM total BtuCD-F), 2 mM luminal ATP, 5 mM MgCl₂ and an ATP-regenerating system. Supplementation with 1 µM additional BtuF is indicated by _addBtuF_. Reducing conditions are shown in green using 10 mM DTT. B12 concentration were 1.5 nM only hot _57Co-B12_ (A) or 1 µM total B12 (B). Empty bars represent samples without luminal ATP. Note the trapped signal indicated with a red arrow.
Figure AII.9: Interpretation of substrate transport by X4wt. Schematic of the different conditions in the transport assay with saturating B12 concentration. The ATP-regenerating system is indicated by A.R.S. A) Covalently attached BtuF feeds the transporter with B12 with a slow transport rates and omission of ATP leads to trapped B12. B) Addition of excess addBtuF does not increase the transport activity under non-reducing conditions, since the transporter is not available for binding of addBtuF. C) Reducing the cross-link leads to about the same transport activity as for the covalently attached BtuF, showing that increasing the local BtuF concentration by cross-linking does not increase the transport activity. D) The presence of an excess amount of B12-loaded BtuF, which can bind and unbind BtuCD, results in the highest transport rates.
Appendix

AII.2.4 Attempts to structural studies of cross-linked BtuCD-F

Previous crystallization experiments with BtuCD-F required concentrated sample around 20 mg/ml [1, 2]. Sample preparation of X4wt and X4, however, started to aggregate at higher concentrations (Fig. AII.10). Due to aggregation at higher concentrations, the cross-linked BtuCD-F was not suitable for crystallization experiments. Nevertheless, structural studies with electron microscopy does not require high sample concentration. Therefore, the cross-linked BtuCD-F sample was reconstituted into amphipols, but due to the lack of available microscope time, this direction was not further followed.

Figure AII.10: Concentrated X4 and X4wt constructs. 500 µl sample was injected on a superdex 200 10/300 GL column equilibrated with 10 mM Tris-Cl pH 7.5, 100 mM NaCl, 0.5 mM EDTA, NaOH pH 8 and 0.01 % (v/v) C12E8. Shown are sample preparations of X4wt at 12.2 mg/ml (A) and X4 at 10.5 mg/ml (B). C) X4wt reconstitution into amphipols. 500 µl of 6 mg/ml sample were injected on a superdex 200 10/300 GL column equilibrated with 20 mM HEPES pH 7.2 and 100 mM NaCl.
References