Investigation of the Vitamin $B_{12}$ Translocation Mechanism for the *Escherichia coli* ABC Importer BtuCD-F using EPR Spectroscopy

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY
ZURICH

for the degree of
Dr. sc. ETH Zurich

presented by

BENESH JOSEPH
M. Eng. Biotechnology
Osaka University, Japan

BORN 29.05.1980
Citizen of India

accepted on the recommendation of

Prof. Dr. Gunnar Jeschke, examiner
Prof. Dr. Kaspar P Locher, co-examiner
Dr. Enrica Bordignon, co-examiner

2013
dedicated to my mother
# Contents

Symbols and Abbreviation ................................................................................. 4  
Abstract ............................................................................................................. 7  
Zusammenfassung ................................................................................................. 9  
1. Introduction .................................................................................................... 11  
   1.1 The ATP Binding Cassette (ABC) Superfamily ........................................... 11  
   1.2 Structure of ABC transporters .................................................................... 12  
      1.2.1 Nucleotide binding domains ................................................................. 13  
      1.2.2 Transmembrane domains .................................................................... 15  
   1.3 Vitamin B$_{12}$ transport in *Escherichia coli* ............................................. 16  
      1.3.1 *E. coli* BtuCD-F complex .................................................................. 17  
   1.4 Substrate translocation mechanism ............................................................... 19  
   1.5 Aim of the thesis ......................................................................................... 20  
2. EPR spectroscopy ............................................................................................. 23  
   2.1 Spin Hamiltonian ......................................................................................... 23  
      2.1.1 Electron Zeeman Interaction ................................................................. 23  
      2.1.2 Nuclear Zeeman Interaction ................................................................ 24  
      2.1.3 Hyperfine Interaction .......................................................................... 25  
      2.1.4 Zero-field splitting .............................................................................. 26  
      2.1.5 Spin-spin interaction .......................................................................... 27  
   2.2 Site-directed spin labeling technique ............................................................. 27  
   2.3 The nitroxide spectrum: continuous wave EPR .......................................... 29  
   2.4 Double Electron Electron Resonance (DEER) ............................................. 32  
      2.4.1 Sensitivity enhancement of DEER experiment ....................................... 34  
      2.4.2 Data analysis ....................................................................................... 35  
   2.5 DEER employing Gd(III)-NO spin pairs ..................................................... 37  
3. Transmembrane gate movements in the type II ABC importer BtuCD-F during nucleotide cycle ................................................................. 39  
   3.1 Introduction .................................................................................................. 39  
   3.2 Experimental procedures ............................................................................ 41
4. Conformational transitions of the vitamin B₁₂ ABC importer in liposomes in the course of substrate translocation

4.1 Introduction .................................................. 62
4.2 Materials and Methods............................................. 64
  4.2.1 Protein Expression and Purification ......................... 64
  4.2.2 Spin labeling and ATP-ase assay .......................... 64
  4.2.3 Liposome Reconstitution of BtuCD and BtuCD-F.............. 64
  4.2.4 SDS-PAGE for observation of complex formation ............ 65
  4.2.5 Vitamin B₁₂ trapping in proteoliposomes ................... 65
  4.2.6 Sample preparation for EPR measurements .................. 65
  4.2.7 EPR Measurements......................................... 66
4.3 Results .................................................................. 67
  4.3.1 Conformation of the translocation channel in BtuCD-F in the absence of nucleotides 67
  4.3.2 ATP binding at NBDs induces major conformational changes in the translocation channel .... 69
  4.3.3 ATP-hydrolysis restores the apo-like conformation of the translocation channel ....... 70
  4.3.4 Response of BtuCD to nucleotide cycle ........................ 71
  4.3.5 Trapping of vitamin B₁₂ following BtuF-BtuCD association .................... 72
  4.3.6 Vitamin and nucleotide effects on BtuCD-F complex formation and conformation of translocation channel ........................................... 72
4.4 Discussion ....................................................... 75
4.5 Supporting Information ........................................................................................................ 78
4.6 Supporting table ...................................................................................................................... 93
5. Summary and Outlook............................................................................................................. 94
A Appendix................................................................................................................................... 97
Bibliography ................................................................................................................................ 100
List of Publications ..................................................................................................................... 111
Acknowledgements ..................................................................................................................... 113
Curriculum Vitae .......................................................................................................................... 114
Symbols and Abbreviation

Symbols

\( A_k \)  
hyperfine tensor of nucleus \( k \)

\( \overrightarrow{B}_0^T \)  
static magnetic field vector (transposed)

\( B(t) \)  
background function of dipolar evolution

\( C_{opt} \)  
optimal spin concentration

\( D \)  
zero-field splitting tensor

\( D_{DD} \)  
dipole-dipole tensor

\( f_{L,\pi} \)  
fraction of spins excited by the observer \( \pi \) pulse

\( F(t) \)  
form factor

\( g \)  
g tensor

\( \text{Gd(III)} \)  
gadolinium ion

\( g_e \)  
g value of the free electron

\( \mathcal{H} \)  
Hamiltonian

\( \hbar \)  
Planck’s quantum of action

\( g_{\text{eff}} \)  
effective g value of the electron

\( \mathcal{H}_0 \)  
static spin Hamiltonian

\( \mathcal{R}_{ee} \)  
electron-electron interaction Hamiltonian

\( \mathcal{R}_{EZ} \)  
electron Zeeman Hamiltonian

\( hf \)  
hyperfine

\( \mathcal{R}_{HF} \)  
hyperfine interaction Hamiltonian

\( \mathcal{R}_{NZ} \)  
nuclear Zeeman Hamiltonian

\( \mathcal{R}_{ZFS} \)  
zero-field splitting Hamiltonian

\( \lambda \)  
inversion efficiency
\( \hat{I}_k \) nuclear spin operator

\( J \) J coupling

\( \mu_0 \) permeability of vacuum

\( \mu_B \) Bohr magneton

\( \mu_n \) nuclear magneton

\( r \) inter-spin distance

\( S \) electron spin vector operator

\( T_1 \) longitudinal relaxation time

\( T_2 \) transverse relaxation time

\( T_{DD} \) dipolar hyperfine tensor

\( T_m \) phase memory time

\( t_{\text{max}} \) maximum dipolar evolution time

\( t_{\text{total}} \) total dipolar evolution time

\( \omega_{dd} \) dipolar frequency

Abbreviations

Å Angstroem

ABC ATP-binding cassette

ADP Adenosine diphosphate

AMPPNP 5’-Adenylylimidodiphosphate

ATP Adenosine triphosphate

CW Continuous wave

Cw continuous wave

DEER double electron-electron resonance

DTPA diethylenetriaminepentaacetic acid

DTT Dithiothreitol

EDTA Ethylenediaminetetraacetic acid

e.g. exempli gratia [Latin: for example]

EPR Electron Paramagnetic Resonance

EZI electron Zeeman interaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton (1000 g mol⁻¹)</td>
</tr>
<tr>
<td>LDAO</td>
<td>N-dodecyl-N,N-dimethylamine-N-oxide</td>
</tr>
<tr>
<td>MTSSL</td>
<td>(1-oxyl-2,2,5,5,-tetramethyl-d-3-pyrroline-3-methyl) methanethiosulfonate spin label</td>
</tr>
<tr>
<td>m.w.</td>
<td>microwave</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide-binding domain</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>NO</td>
<td>nitroxide</td>
</tr>
<tr>
<td>NZI</td>
<td>nuclear Zeeman interaction</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PELDOR</td>
<td>pulsed electron double resonance</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein (MDR1)</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDSL</td>
<td>site-directed spin labeling</td>
</tr>
<tr>
<td>TEMPO</td>
<td>2,2,6,6-tetramethyl-1-piperidinyloxy</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)-aminomethan</td>
</tr>
<tr>
<td>ZFS</td>
<td>zero-field splitting</td>
</tr>
</tbody>
</table>
Abstract

ATP-binding cassette (ABC) transporters are ubiquitous integral membrane proteins that translocate diverse substrates such as sugars, amino acids, peptides, vitamins, iron siderophores, opines, metals, etc. across cell membranes. Crystallographic and biochemical evidence supports a ‘two-state, alternating-access’ mechanistic model for both ABC exporters and importers. In this model, when ATP is bound to the nucleotide binding domains (NBDs), the transmembrane domains (TMDs) adopt a conformation facing the extracellular side of the membrane, which is converted to an inward-facing conformation via ATP hydrolysis in the NBDs. This conformational transition ensures net substrate uptake by the importers or net expulsion by the exporters. Based on the TMD architecture, the ABC importers are classified into type I and type II. Type I ABC importers contain 12-14 transmembrane helices (e.g. the maltose, methionine and molybdate importers), whereas type II ABC importers contain 20 transmembrane helices (e.g. the vitamin B_{12} and the heme importers). There is growing evidence showing that the structural differences between type I and type II ABC importers are accompanied with mechanistic diversity in substrate translocation.

The high resolution crystal structures obtained with several ABC transporters in different steps of the transport cycle have greatly enhanced our understanding of the transport mechanism. However for an ABC transporter, a complete elucidation of the transport cycle requires crystal structures obtained in the presence of ATP, ATP-VO_{4}^{2-} (transition state), ADP, substrate and when possible both nucleotide(s) and substrate. For an ABC importer like BtuCD-F even more conformational intermediates are possible due to the presence of an additional periplasmic subunit, namely the substrate binding protein (BtuF). With the notable exception of the type I maltose importer MalFGK_{2}-E, it has not yet been possible with most of the ABC transporters to get the crystal structures for the majority of the possible intermediates of the transport cycle. Moreover, with the crystal structures obtained using detergent-solubilized transporters, it remains to be verified whether the observed conformations (in the absence of a lipid bilayer membrane) represent physiologically relevant intermediates. There is lack of complementary techniques to bridge the structural information obtained from crystal structures to that in a membrane environment. In this thesis work I used site-directed spin labeling (SDSL) combined with Double Electron Electron Resonance (DEER) to investigate the conformational changes of BtuCD and BtuCD-F under conditions close to the physiologically relevant membrane environment. DEER is an emerging, powerful tool to obtain interspin distances, thus structural information on membrane proteins. Introducing nitroxide-based spin labels at key positions in the translocation channel of BtuCD and BtuCD-F, I studied the
conformation of the translocation channel in the six possible intermediate stages of the transport cycle (apo-, ADP- and ATP-states in BtuCD and BtuCD-F) in liposome-reconstituted transporters. In addition, I monitored the effects induced by substrate and nucleotides on the conformation of the translocation channel and on the extent of complex formation between BtuCD and the substrate binding protein BtuF.

Comparison of the experimental DEER data with simulations performed on the available crystal structures of apo-BtuCD, apo-BtuCD-F and AMPPNP-BtuCD-F revealed good agreement for key gate positions along the translocation channel. ATP binding to liposome-reconstituted BtuCD-F induced conformational changes of the key gate positions along the translocation channel as observed in the crystal structures. In the absence of the binding protein BtuF (BtuCD alone), such conformational changes were not present during ATP binding and hydrolysis. DEER restraints suggest that in liposomes the translocation channel in both apo-BtuCD-F and Mg²⁺-ADP-BtuCD-F has an occluded conformation as observed in the apo-BtuCD-F crystal structure and does not have space to accommodate a vitamin B₁₂ molecule. Interestingly, we found that the interaction of vitamin-loaded BtuF with liposome-reconstituted BtuCD in the absence of nucleotides or in the presence of Mg²⁺-ADP resulted in non-productive release of vitamin B₁₂ in the supernatant. Only in the presence of AMPPNP vitamin B₁₂ could be trapped in the liposome fraction in agreement with the recent findings by Korkhov et al. (2012). To follow simultaneously the conformation of the translocation channel and the BtuCD-BtuF complex formation, a Gd(III)-based label was attached to BtuF and nitroxide-based labels to BtuC. The Gd(III)-NO experiment has already been tested on model systems and soluble proteins and in this work it was applied for the first time to a membrane protein. Selective Gd(III)-nitroxide and nitroxide-nitroxide distance measurements with liposome reconstituted BtuCD-F showed that the AMPPNP-bound BtuCD-F complex exists in liposomes with a sealed cavity along the translocation channel as observed in the crystal structure. The vitamin B₁₂ is most likely trapped in the translocation channel in this state and our data suggests that it may not modify the sealed conformation of the cavity. Vanadate trapping experiments suggest that the substrate can be released during conversion from the transition state intermediate to the Mg²⁺-ADP-bound form of BtuCD-F. The EPR and biochemical data obtained in this thesis, combined with the three available crystal structures of this type II importer provided new insights into the vitamin B₁₂ transport mechanism in E. coli.
Zusammenfassung


1. Introduction

Cells are surrounded by a membrane bilayer, which restricts the movement of solutes between the exterior and the cytoplasm except for gases and small-uncharged molecules. Membrane transport proteins facilitate the movement of specific molecules across the membrane. Thus the cell membrane is equipped with diverse transport proteins for different molecules involved in physiology and metabolism. Molecules like glucose or amino acids with large (electro)chemical gradient across the membrane can be transported as such by special carrier proteins. This is called a passive transport as the process is thermodynamically driven by an increase in entropy and decrease in free energy. For several molecules the transport has to be achieved against a concentration gradient and it requires a membrane transporter and an energy source. These membrane proteins are called primary active transporters if they use ATP or secondary active transporters if they use an electrochemical gradient as the energy source to drive the transport process. In this thesis work, I investigated the transport mechanism for the primary active ATP-Binding Cassette (ABC) transporter BtuCD-F mediating vitamin B\textsubscript{12} uptake in \textit{E. coli}.

1.1 The ATP Binding Cassette (ABC) Superfamily

ABC-systems are found in all three domains of life and form one of the largest protein superfamilies of paralogous sequences [1, 2]. This superfamily contains two subfamilies. The largest subfamily contains the primary active transporters mediating the transport of diverse substances across the membrane. The second subfamily contains non-transporting ABC proteins involved in DNA repair, maintenance and gene regulation [2]. On comparison of genome size, bacteria contain the largest number of ABC-systems, highlighting their importance for bacterial survival including that of several pathogens. \textit{E. coli} contains 57 ABC transporters, whereas the human genome contains 48 genes for ABC transporters [3, 4].

ABC transporters are involved in transport of diverse substances such as sugars, amino acids, vitamins, metal chelate complexes, peptides, lipids, drugs, proteins and polysaccharides. Depending on the directionality of the transport, ABC transporters are further classified into importers or exporters. In bacteria, ABC importers mediate the uptake of important nutrients, like the maltose importer MalFGK\textsubscript{2}-E and the vitamin B\textsubscript{12} importer BtuCD-F in \textit{E. coli} [1]. ABC exporters form part of protein or lipid (e.g. MsbA [5]) secretion systems or confer resistance to toxins or antibiotics (like LmrA [6] in \textit{Lactococcus lactis} and Sav1866 [7] of \textit{Staphylococcus aureus}) by actively pumping them out. A recent classification based on sequence homology of ABC domains revealed that ABC systems
diverged very early during evolution into three functional groups, i.e., importers, exporters and others [1].

1.2 Structure of ABC transporters

The general architecture of an ABC transporter consists of two transmembrane domains (TMD) connected to two cytosolic nucleotide-binding domains (NBDs). There exists significant diversity in sequence and structure for TMDs, reflecting the specificity for diverse substrates. NBDs have conserved sequences and structural elements. In case of bacterial importers, these four domains consist of independent polypeptides (figure 1.1).

Figure 1.1: Domain architecture in ABC transporters. **Left**) NBDs and TMDs are formed from four independent polypeptides, e.g. the vitamin B₁₂ importer BtuC₂D₂-F from *E. coli* [8]. The substrate binding protein BtuF is shown in blue. The substrate binding protein is anchored to the cytoplasmic membrane in Gram-positive bacteria and Archaea. **Middle**) One NBD and one TMD are fused to form a half transporter and two of them make a homodimer to form a full transporter (e.g. MsbA and human TAP1/2). In the case of TM287-TM288 from *Thermotoga maritima*, the functional transporter is a heterodimer. **Right**) A single polypeptide contains two NBDs and two TMDs as found in LmrA and P-glycoprotein.

With most of the importers, a substrate binding protein (SBP) is required to bind and deliver the substrate to TMDs. In case of exporters, these domains may consist of a homo- (for e.g. Sav1866 and MsbA) or hetero (TM287-TM288 [9]) dimer or just one polypeptide (for e.g. LmrA and P-glycoprotein[10]). Only ABC exporters are present in eukaryotes. In humans, ABC transporters are associated with many diseases like
1.2 Structure of ABC transporters

Adenoleukodystrophy (ALD) and Stargardt’s disease (hABCR). Overexpression of P-gp or MRP1 confers multidrug resistance in mammalian cancer cells [11]. Two other examples of important humans ABCs are the CFTR gene, which in a mutated form is responsible for cystic fibrosis and TAP associated with antigen processing and presentation to major histocompatibility complex [12].

1.2.1 Nucleotide binding domains

ABC transporters harness the energy of ATP hydrolysis to the net transport of substrates. The NBDs are analogous to the engine of these small molecular machines. NBDs bind and hydrolyze ATP to induce conformational transitions of TMDs responsible for substrate translocation. Despite the diversity of the TMDs connected with, NBDs have a similar architecture and conserved structural elements among all the ABC transporters (figure 1.2).

![Figure 1.2: Structural elements of NBDs. A) A schematic overview of conserved sequences among all the ABCs. B) The ABC homodimer viewed down from the local twofold axis of symmetry. The RecA-like subdomains are shown in green and the helical subdomain in cyan. ATP (in ball and stick representation) is sandwiched between the “Walker A”-motif (WA, red) of one monomer and the LSGGQ-signature sequence (magenta) of the other monomer. The “Walker-B”-motif is shown in blue and the Q-loop in yellow. Adapted from [1, 13] The NBDs consist of a larger RecA-like domain consisting of β-sheets and α-helices connected to a smaller helical domain consisting of 3-5 α-helices. The RecA-like domain contains the conserved Walker A motif with the sequence GxxGxGKS/T, where x is any amino acid and the Walker B motif with the sequence φφφφD, where φ is a hydrophobic residue. The helical domain contains the LSGGQ motif, also known as the signature motif unique to ABC proteins. The Rec-A like and the helical domain are connected with two
flexible loops. One of these loops known as the Q loop contains a highly conserved glutamine residue and it is important for mediating interactions of the NBDs with the TM subunits in intact transporters [7, 14-16].

ATP hydrolysis by NBDs requires dimerization and in all the known ABC transporter structures NBDs exist as a dimer. In these structures, ATP is bound between the Walker A motif of one subunit and the LSGGQ motif of the other (figure 1.2). The adenosine ring of the ATP is stabilized through a ring-stacking interaction with a conserved aromatic amino acid preceding the Walker A motif. The Walker A motif forms a loop that binds with the α- and β- phosphate groups. The terminal aspartate of the β-stranded Walker B motif is important to coordinate Mg²⁺ ions required for ATP hydrolysis. The H loop, which contains a conserved histidine forms a hydrogen bond with the γ-phosphate and is required for hydrolysis [17, 18]. On the other side, the LSGGQ motif coordinates the γ-phosphate through the side chain of the serine and the backbone amide groups of the glycine residues. The precise mechanism of ATP hydrolysis is still not very clear. A glutamate residue at the end of the Walker B motif is proposed to act as a general base to polarize the water molecule attacking the β-phosphate group. However, data obtained with Pgp, HlyB, and GlcV [18-21] suggests a substrate-assisted catalysis mechanism for ATP hydrolysis, where the histidine in the H-loop plays a greater role and the Walker B glutamate is needed to restrict the flexibility of the H loop for it to adopt a catalytically competent conformation.

Crystal structures of MalK [22] and HlyB [18] in multiple liganded states have revealed the structural basis of ATP binding and hydrolysis. A motion in a tweezers-like fashion where the NBDs close and open like the tips of a pair of tweezers upon binding and hydrolysis of two ATP molecules has been revealed from these crystal structures. The ADP-state has a semi-open conformation, suggesting a restoration of the fully open apo-state following ADP dissociation [22].

Available biochemical and structural data point positive cooperativity in ATP binding [1, 17] at NBDs. Data on maltose and OpuA systems suggests that ATP hydrolysis at both sites might be important for function [23, 24]. However, the sequence of ATP hydrolysis or the stoichiometry of hydrolysis per transport cycle is not yet convincingly proven. The existing data on the number of ATP molecules used per substrate transported is controversial even for the same transporter and such assays in general are complicated with problems like uncoupled ATP hydrolysis or substrate leakage [1]. Vanadate trapping experiments with P-glycoprotein suggests that hydrolysis occurs alternatively and one ATP is used per molecule of drug transported [25, 26]. An alternate progressive clamp model has been proposed for the mitochondrial ABC transporter Mdl1p [27], where two ATPs are hydrolyzed sequentially before the NBDs separate. Indeed more studies are required to reveal the sequence of ATP hydrolysis. Considering the structural diversity among ABC transporters (homo and heterodimeric ABC transporters and CFTR with heterodimeric NBDs) it is possible that more than one mechanism exits.
1.2 Structure of ABC transporters

1.2.2 Transmembrane domains

So far, twelve different ABC transporters (seven importers and five exporters) have been crystallized [5, 7, 9, 10, 14-16, 28-34], with some of them trapped in multiple intermediate conformations during the translocation cycle. The extensively studied maltose importer has been crystalized in several of the possible intermediates of the transport cycle [35-37]. The available crystal structures revealed a common architecture for ABC transporters consisting of two TMDs connected to two cytosolic NBDs. However, despite the common architecture, the crystal structures revealed significant differences, especially for the topology of the TMDs (figure 1.3).

![Figure 1.3: Schematic views of the topology of a TM subunit. Left) Schematic of the ABC exporter Sav1866 from S. aureus showing the pseudo two fold symmetry relating helices TM1-3 to TM4-6. Middle) Topology of ModB subunit of the ABC type I importer ModBC from A. fulgidus containing 6 TM helices. Right) Topology of a BtuC subunit of the type II ABC importer BtuCD-F for vitamin B12 from E. coli, containing 10 TM helices. Adapted from [38].](image)

In ABC importers, the TMDs consist of a homodimer as in BtuCD or of a heterodimer as in MalFGK2. In bacterial importers, the TMDs contain 5-10 helices per monomer connected by short loops. The interconnecting loops have important functions such as gating along the translocation channel as in BtuCD [39], and interaction or stabilization of interacting proteins. A substrate-binding site in the TMD is identified only in the maltose transporter [28]. However, the crystal structure of AMPPNP-BtuCD-F [40] and the binding protein-independent TMD mutants of the arginine transporter, which can bind substrate [41], suggest the existence of a substrate binding site in TMDs of other transporters as well. All ABC transporters contain two architecturally conserved short α-helices called ‘coupling helices’ interacting via salt bridges with the residues in the Q-loops of the NBDs. Thus, the coupling helices act as the interface for transmitting conformational changes from the NBDs to TMDs. There is substantial biochemical evidence proving that this interaction is essential for coupling between NBDs and TMDs [42-44]. Based on the architecture, ABC importers are classified [45] into type I (maltose, methionine and molybdate systems with 12-14 TM helices) and type II (vitamin B12, Hi1470/71 and heme systems with 20 TM helices).
There is increasing evidence for a type III ABC importer group formed by the energy coupling factor (ECF) transporters mediating the uptake of vitamins and other nutrients needed in trace amount [46, 47]. These importers have an unprecedented architecture consisting of a conserved transmembrane and nucleotide binding domains, together forming an ‘energy coupling module’ [32, 33]. Interestingly, similar to the substrate binding proteins of type I and type II ABC importers, they use small integral membrane proteins to capture specific substrates. Though many substrate capture proteins have a specific energizing module, numerous unrelated substrate capture proteins share the same energy-coupling module.

ABC exporters have 6 helices per monomer forming a conserved TMD consisting of 12 helices. Unlike ABC importers, they do not have a substrate binding protein and recruit the substrate directly from the cytoplasm or from the lipid bilayer [48].

1.3 Vitamin B_{12} transport in *Escherichia coli*

Unlike the gram-positive bacteria, *E. coli* has an outer membrane covering the inner membrane with a space in between called periplasm. Molecules with mass below 600 Da enter the periplasm through non-specific outer membrane proteins called porins. However vitamin B_{12} exceeds this size limit and it is transported into the periplasm by a specific outer membrane protein BtuB at the expense of the cytoplasmic electrochemical gradient [49].

*Figure 1.4*: Schematic view of vitamin B_{12} transport in *E. coli*. The outer membrane protein BtuB binds vitamin with high affinity and release it into the periplasm through a conformational change induced by TonB. The BtuB-TonB interaction is mediated by the ExbB-ExbD complex in the inner membrane, which induces conformational changes in TonB in the presence of a proton motive force. The free vitamin B_{12} in the periplasm is bound by BtuF and delivered to the transmembrane ABC transporter BtuCD, which transports vitamin into the cytoplasm at the expense of ATP binding and hydrolysis. Adapted from [50].

BtuB consists of a 22-stranded β-barrel with an N-terminal globular plug lying inside. The energy transduction to BtuB is achieved by the inner membrane ExbB-ExbD-TonB complex. The periplasmic domain of TonB interacts with a conserved region of BtuB called the TonB box which triggers the release of bound vitaminB_{12} to periplasm. The vitamin B_{12} binding protein BtuF binds the free vitamin in the periplasm with high affinity and delivers it
1.3 Vitamin B\textsubscript{12} transport in \textit{E.coli}

to the type II ABC transporter BtuCD in the inner membrane, which finally imports the vitamin into the cytoplasm at the expense of ATP hydrolysis (figure 1.4).

1.3.1 \textit{E. coli} BtuCD-F complex

The presence of a binding protein for vitamin B\textsubscript{12} in the periplasm suggested from earlier biochemical studies [51, 52] was later confirmed with vitamin B\textsubscript{12} binding studies using purified BtuF [53]. Genetic analysis in \textit{E. coli} revealed the further components BtuC and BtuD in vitamin B\textsubscript{12} uptake [54] and expression studies suggested that BtuCD is localized at the cytoplasmic membrane [55, 56].

\textbf{Figure 1.5}: Crystal structures of BtuCD and BtuCD-F. \textbf{A}) BtuCD (PDB 1L7V) with an outward-facing TMD arrangement. The NBDs do not have any bound nucleotides and are arranged side-by-side with rather small contact surface. The TM5 helices are highlighted in blue. \textbf{B}) BtuCD-F (PDB 2QI9) with an occluded translocation channel. BtuF is shown in red and TM5 helices in blue. NBDs adopt a very similar conformation as observed in BtuCD. \textbf{C, D}) Conformation of TM5 and TM5a helices in BtuCD and BtuCD-F, respectively revealing the asymmetry observed in BtuCD-F. The short loop between TM5 and TM5a at the periplasmic side and that between TM4 and TM5 at the cytoplasmic side act as the gates at the entry and exits points, respectively (highlighted by arrows).

Locher \textit{et. al.} reported the first crystal structure of BtuCD in 2002 [15]. Later his laboratory reported two more crystal structures for BtuCD-F [8] and AMPPNP-BtuCD-F [40] providing further details of the transport mechanism of vitamin B\textsubscript{12} (figure 1.5). In BtuCD, the TMDs are formed by two BtuC subunits and the NBDs are formed by two BtuD subunits. The BtuCD crystal structure revealed outward-facing TMDs with a periplasmic cavity, which could harbor a vitamin B\textsubscript{12} molecule (figure 1.5A). The short loop at the entry point for vitamin B\textsubscript{12} between TM5 and TM5a is called the periplasmic gate and the loop
between TM4 and TM5 at the exit point for vitamin B$_{12}$ is called the cytoplasmic gate (figure 1.5C). The NBDs do not have any bound nucleotides and are arranged side-by-side with rather small contact surface. Two short coupling helices between transmembrane helices TM6 and TM7 make extensive contact with the NBDs mostly with the residues around the Q-loop.

Another type II ABC importer for heme, HmuUV from *Yersinia pestis* was crystallized with a similar conformation [31], supporting the physiological relevance of this structure. Yet, another architecturally related Hi1470/1 transporter from *Haemophilus influenzae* was crystallized with a similar conformation of the NBDs and coupling helices, but with inward-facing TMDs [16].

In the apo-BtuCD-F crystal structure there is no vitamin trapped inside and BtuF adopts a substantially open conformation with the periplasmic BtuC loops (periplasmic gate) inserted into the vitamin B$_{12}$ binding pocket (figure 1.5B). Interestingly, the TMDs revealed substantial asymmetry especially at the cytoplasmic side (figure 1.5D). The orientation of the helices TM3-TM5a determines to which side of the membrane the translocation pathway is open. The asymmetric orientation of these helices in the two BtuC subunits makes the translocation pathway occluded and too small to harbor a vitamin B$_{12}$ molecule. The NBDs adopt a conformation very similar to that observed in BtuCD; however, the coupling helices are closer as compared to those in BtuCD or Hi1470/1.

During my PhD thesis work, BtuCD-F was crystallized by Korkhov et al. with the non-hydrolysable ATP analogue AMPPNP [40] using the ATP-ase deficient E159Q mutant stabilized with cysteine cross-linking (N162C) in BtuD (figure 1.6). This structure revealed that the AMPPNP-induced closure of the NBDs brings the coupling helices closer and opens the TM5 helices of the translocation channel towards the cytoplasm.

During my PhD thesis work, BtuCD-F was crystallized by Korkhov et al. with the non-hydrolysable ATP analogue AMPPNP [40] using the ATP-ase deficient E159Q mutant stabilized with cysteine cross-linking (N162C) in BtuD (figure 1.6). This structure revealed that the AMPPNP-induced closure of the NBDs brings the coupling helices closer and opens the TM5 helices of the translocation channel towards the cytoplasm.

**Figure 1.6:** AMPPNP-BtuCD-F crystal structure (PDB 4FI3). The TM5 and TM10 helices along the translocation channel are highlighted in green and red colors, respectively. The short loop between TM2 and TM3 called the cytoplasmic gate II, which seals the translocation pathway shut is highlighted in gold. Also shown is an enlarged view of the translocation channel with the proposed cavity that traps vitamin in the ATP-state.
1.4 Substrate translocation mechanism

Unexpectedly, the loop connecting TM2 and TM3 helices moved into the opening made by the swing-out motion of the TM5 helices creating a sealed cavity along the translocation channel. Sequence alignment suggest that these loops called the ‘cytoplasmic gate II’ might be conserved among type II ABC importers [40]. Complemented with substrate trapping experiments in liposomes, it is proposed that in the ATP-state vitamin B$_{12}$ is trapped in the cavity along the translocation channel in BtuCD-F and that vitamin could be released following ATP hydrolysis through an inward-facing conformation similar to the one observed in Hi1470/1.

1.4 Substrate translocation mechanism

It was proposed that a general coupling mechanism may allow both ABC importers and exporters to convert ATP binding and hydrolysis to conformational changes that facilitate active transport [45]. The type I maltose importer MalFGK$_{2}$-E has been crystallized in several intermediate states of the translocation cycle. Analysis of these structures and that of another type I ABC importer ModBC-A from Archaeoglobus fulgidus [14] validates an ‘alternating access’- model for substrate translocation (figure 1.7). In this model, ATP-induced closure of the NBDs exposes the translocation pathway to the periplasm and following ATP hydrolysis the TMDs restore towards an inward-facing conformation. The crystal structure of the Staphylococcus aureus multidrug exporter Sav1866 with outward-facing TMDs in the ATP-state and that of the mouse P-glycoprotein with inward facing TMDs in the absence of any nucleotides at the NBDs suggest that ABC exporters as well function with a similar mechanism. For importers, in the outward-facing conformation substrate enters in the translocation pathway while for exporters the substrate is released.

Intriguingly, for type II ABC importers, the crystal structures of BtuCD, BtuCD-F, Hi1470/1 and HmuUV did not completely reconcile with the alternating access- model and it was proposed that these transporters may function with a different mechanism [45]. It was shown that ATP enhances BtuCD-F complex dissociation, and in the presence of ATP and vitamin the dissociation is accelerated by several orders of magnitude [58], different from what was shown in type I importers. Thus a mechanism in which vitamin B$_{12}$ is directly translocated in the apo-state and ATP binding is required only for BtuF dissociation was proposed.

Interestingly, the recent AMPPNP-BtuCD-F structure revealed a very distinct conformational coupling between NBDs and TMDs [40] and has shown the existence of a cavity along the translocation channel (figure 1.6), which could harbor a vitamin molecule. Based on this structure and on vitamin trapping experiments, it was suggested that in the ATP-state, BtuCD-F traps the vitamin in the translocation channel and that vitamin is released following ATP-hydrolysis. In this thesis work I use EPR spectroscopy on the
membrane reconstituted transporter to follow the conformational changes during vitamin transport and to bridge the “mechanistic” gap between the observed enhanced complex dissociation in the presence of vitamin and ATP and the trapping of vitamin in BtuCD-F under the same conditions during a translocation cycle.

**Figure 1.7:** Translocation mechanism for type I ABC importers. In the absence of ATP, the NBDs are open and the transporter adopts an inward-facing conformation. Interaction of substrate-binding protein in the ATP-state induces an outward-facing conformation of TMDs and the substrate is released from the binding protein. Following ATP-hydrolysis, NBDs open and TMDs restore to an inward-facing conformation to complete substrate translocation. Adapted from [57].

1.5 Aim of the thesis

At the beginning of this thesis work, two crystal structures were available (apo-BtuCD and apo-BtuCD-F) for the vitamin B_{12} importer. Unlike the outward-facing BtuCD structure, the architecturally related Hi1470/1 was crystallized with an inward-facing conformation. Based on the first BtuCD crystal structure, it was proposed that ATP-induced closure of NBDs would couple the TMDs to an inward-facing conformation [15], opposite to that of type I ABC importers. However, the BtuCD-F crystal structure with an occluded translocation channel, which was thought to be an intermediate-structure following vitamin B_{12} release suggested that BtuCD-F as well might import vitamin with a similar mechanism as in type I ABC importers [8].

This thesis is aimed to obtain a deeper understanding of the conformational changes in BtuCD during vitamin B_{12} import in a membrane environment. Though crystal structures are indispensable for mechanistic understanding of ABC transporters, they provide only few snapshots of selected conformational intermediates. In addition, detergent solubilization removes the natural membrane environment, which can lead to changes in the conformation of the protein [45, 59]. As the current understanding of vitamin B_{12} translocation mechanism is mostly based on crystal structures, it is important to verify that the crystal structures represent relevant intermediates of the transport cycle in a
membrane environment. A part of this thesis work involves comparative structural analysis of BtuCD and BtuCD-F in LDAO detergent micelles and liposomes using site-directed spin labeling (SDSL) and Electron Paramagnetic Resonance (EPR) spectroscopy. The experimental EPR measurements were compared with simulations performed on the available crystal structures to reveal the effect of environment if any, on the conformation of the complex.

To better understand the transport mechanism, we need structural information for several possible intermediates in the transport cycle. However, membrane proteins including ABC transporters are very dynamic in nature and they do not easily form a three-dimensional crystal lattice [45]. Site-Directed Spin Labeling (SDSL) combined with Electron Paramagnetic Resonance (EPR) is a powerful tool to obtain structural information on membrane proteins like ABC transporters in a native-like membrane environment [39, 60-63]. Using SDSL-EPR with membrane reconstituted BtuCD and BtuCD-F, I obtained structural information for several intermediates to delineate the possible sequence of events and the conformational changes in a transport cycle. Further, I studied the effects of nucleotides and/or vitamin B_{12} on BtuCD-F complex stability and on the conformation of the translocation channel in liposomes. In this thesis I present for the first time application of orthogonal spin-labeling and selective measurement of two distances (in the same sample) between different spin labels in a membrane protein complex using Double Electron Electron Resonance (DEER). BtuF and BtuCD were labeled with Gd(III) and nitroxide (MTSSL, referred as NO) spin labels, respectively. Selective measurements of Gd(III) - NO and NO - NO distances on the same sample were performed to follow complex formation and conformation of the translocation channel. These results were complemented with biochemical studies to follow vitamin B_{12} trapping upon interaction of vitamin-loaded BtuF with BtuCD in presence or absence of nucleotides in liposomes.

The early results obtained in this PhD thesis work on the conformation of the periplasmic and the cytoplasmic gates on the TM5 helices during ATP binding and hydrolysis in liposomes were published in 2011 [39] and are shown in chapter III. There I show that in the ATP-state of the BtuCD-F complex, the periplasmic gate closes and the cytoplasmic gate opens, suggesting an inward-facing pathway for vitamin release to the cytoplasm.

One year after this publication, the AMPPNP-BtuCD-F structure was published by Korkhov et.al. [40], which confirmed the closure and opening of the periplasmic and cytoplasmic gates, respectively, as I reported before. However, the translocation channel was found to have a cavity, which could possibly harbor a vitamin molecule. The diffraction data obtained with the ATP-ase deficient E159Q mutant stabilized with cysteine cross-linking at the NBDs does not reveal electron density for a vitamin molecule. In chapter IV, I present a detailed analysis of the conformation of the translocation channel in liposomes as influenced by the presence of vitamin and or nucleotides, which has been submitted for publication.
The results presented in this thesis allow a detailed understanding of the conformational changes and complex dynamics in BtuCD-F, associated with ATP-dependent vitamin $\text{B}_{12}$ import in a membrane environment. The results confirm the mechanistic diversity of BtuCD-F compared to the type I importer MalFGK$_2$-E in terms of substrate binding protein-transporter interaction, NBDs-TMDs coupling and provide new insights into the mechanism of vitamin $\text{B}_{12}$ import, all in a membrane environment.
2. EPR spectroscopy

2.1 Spin Hamiltonian

The theoretical background of EPR spectroscopy is well established and extensively detailed in several textbooks \([64-66]\). Here a brief quantum mechanical description necessary for the understanding of the nitroxide spectrum is provided, which is relevant for the understanding of the EPR experiments presented in this thesis.

The static spin Hamiltonian in equation 2.1 \((\hat{H}_0)\) which describes the energy levels of an unpaired electron with spin \(S\) surrounded by \(n\) nuclei with spin \(I_k\) is a combination of different terms: the electron Zeeman interaction \((\hat{H}_{\text{EZ}})\), the nuclear Zeeman interaction \((\hat{H}_{\text{NZ}})\), the hyperfine coupling between electron and nuclear spins \((\hat{H}_{\text{HF}})\), the zero-field splitting \((\hat{H}_{\text{ZFS}})\), and the interaction between two electrons \((\hat{H}_{\text{ee}})\).

\[
\hat{H}_0 = \hat{H}_{\text{EZ}} + \hat{H}_{\text{NZ}} + \hat{H}_{\text{HF}} + \hat{H}_{\text{ZFS}} + \hat{H}_{\text{ee}} \tag{2.1}
\]

The magnitude of the electron and nuclear Zeeman interactions are dependent on the external magnetic field. Thus, it is possible to resolve the different interactions in the static spin Hamiltonian by performing EPR experiments at different magnetic fields.

2.1.1 Electron Zeeman Interaction

The interaction between one electron spin and the external magnetic field is expressed by the electron Zeeman interaction (EZI) term given by

\[
\hat{H}_{\text{EZ}} = \frac{\mu_B}{\hbar} \vec{B}^T \cdot \vec{g} \cdot \hat{S} \tag{2.2}
\]

Where \(\mu_B\) is the Bohr magneton, \(\hbar\) is the reduced Planck constant, \(\vec{B}^T\) is the transpose of the magnetic field vector, \(\hat{S}\) is the electron spin vector operator and \(\vec{g}\) can be a tensor, which contains the orientation dependence of the interaction, or an effective \(g\) value. If we consider an effective \(g\) value and a spin \(S = \frac{1}{2}\) system (as in the case of the nitroxide radical), the eigenvalues of this Hamiltonian operator gives the energy of the two spin states as follows,
Chapter 2. EPR spectroscopy

\[ E = \pm \frac{1}{2} g \mu_B B_0 \]  \hspace{1cm} (2.3)

**Figure 2.1:** Zeeman splitting of energy levels in an \( S = \frac{1}{2} \) spin system. The splitting of the energy levels with increasing magnetic field is shown in blue. The Boltzmann population difference at the same temperature is increased at higher frequencies. The transitions between the two populated energy states can be induced by microwave irradiation.

For a free electron \( g_e \approx 2.0023 \). The deviation from the free electron value and the orientation dependence of the EZI term for unpaired electrons localized in atomic or molecular orbitals is due to the spin orbit interaction, caused by the interaction between ground and excited states. For most organic radicals the deviations are rather small (\( \Delta g \approx 0.1 \)). If the g tensor is visualized by an ellipsoid with orthorhombic symmetry (\( g_x \neq g_y \neq g_z \)), with the direction of the magnetic field \( B_0 \) given by the polar angles \( \theta \) and \( \phi \), the effective g value for a particular orientation is expressed as

\[ g_{eff} = (g_x^2 \sin^2 \theta \cos^2 \phi + g_y^2 \sin^2 \theta \sin^2 \phi + g_z^2 \cos^2 \theta)^{1/2} \]  \hspace{1cm} (2.4)

Under the high-field approximation, where the electron Zeeman interaction dominates all other interactions in the static spin Hamiltonian (equation 2.1), the resonance position at this orientation of the molecule is given by

\[ B_{0, res} = \frac{E}{g_{eff} \mu_B} = \frac{h \nu}{g_{eff} \mu_B} \]  \hspace{1cm} (2.5)

As an example, the EPR spectrum of a powder sample with axially symmetric g tensor, with \( g_\perp = g_x = g_y \) and \( g_\parallel = g_z \) containing random orientations of the paramagnetic spin systems with respect to the external magnetic field \( B_0 \) has a line shape as shown in figure 2.2.

2.1.2 Nuclear Zeeman Interaction

Similar to the previously described interaction between electron spins and an external
magnetic field, the energy levels of nuclear spin are as well quantized due to nuclear Zeeman interaction (NZI) term, expressed as

\[ \mathcal{H}_{NZ} = -g_n \frac{\mu_n}{\hbar} B_0 I_{zk} \]  

(2.6)

Figure 2.2: Visualization of an axial g ellipsoid. Left) The direction of the static magnetic field \( \mathbf{B}_0 \) is specified by the angle \( \theta \). Right) The powder spectrum from a sample containing randomly oriented individual spin packets. Adapted from [67].

with \( g_n \) being the nuclear g value, \( \mu_n \) the nuclear magneton and \( I_{zk} \) the nuclear spin operator \( I_z \) for spin \( k \). In general this term is neglected when computing EPR spectra as the magnetic moment of electrons is about 660 times higher than that of hydrogen and even larger factor applies for other nuclei. The \( g_n \) factor is dimensionless and like the nuclear spin quantum number it is an inherent property of the nucleus. For a given value of \( I \), a splitting into \( 2I + 1 \) energy levels are observed with each level characterized by a nuclear magnetic spin quantum number \( m_I = -I, -I + 1, \ldots, I - 1, I \).

2.1.3 Hyperfine interaction

When the electron spin \( S \) is coupled to nuclear spins \( I_k \), the interaction is described by the Hamiltonian for the hyperfine interaction (HF) term, given as

\[ \mathcal{H}_{HF} = \sum_{k=1}^{n} S^T A_k I_k \]  

(2.7)

Where \( A_k \) is the hyperfine tensor, which can be written as the sum of two interactions- the isotropic Fermi contact interaction (FC) and the anisotropic electron-nuclear dipole-dipole coupling (DD). The FC interaction depends on the electron spin density at the nucleus. Thus for an unpaired electron in any orbitals except for the s-orbitals (p, d, f orbitals), there will be no isotropic interaction (\( a_{iso} \)) with the nucleus, since these orbitals have a node at the site of nucleus. The Hamiltonian for the FC interaction is given by

\[ \mathcal{H}_{HF-FC} = a_{iso} S^T I_k \]  

(2.8)

where the isotropic hyperfine coupling constant \( a_{iso} \) is given by,
Chapter 2. EPR spectroscopy

\[ a_{iso} = \frac{2 \mu_0}{3} g_e \mu_B g_n \mu_n |\Psi_0(0)|^2 \]  

(2.9)

with \( \mu_0 \) representing the permeability of the vacuum and \( |\Psi_0(0)|^2 \) giving the electron density at the nucleus. The anisotropic dipolar part of the hyperfine interaction stems from the orientation dependent interaction of the magnetic moments of the electron and of the nucleus and it is given as

\[ \hat{\mathcal{H}}_{HF-DD} = \frac{\mu_0}{4\pi \hbar} g_e \mu_B g_n \mu_n \left( \frac{(3 \mu^T \hat{r})}{r^5} - \frac{\mu^T \hat{r}}{r^3} \right) = \mu^T T_{DD} \mu_k \]  

(2.10)

with the distance between the electron and the nucleus given by \( r \) and \( T_{DD} \) representing the dipolar hyperfine tensor. If spatial distribution of the unpaired electron is significant on the length scale of \( r \), the interaction is an average over all significant centers of spin density, or, more precisely, its spatial integral over electron spin density.

2.1.4 Zero-field splitting

The splitting of the energy levels for spin systems with group spin \( S > 1/2 \) in the absence of any external magnetic field is called zero-field splitting (ZFS). It occurs for example in transition metals having more than one unpaired electron. The strong coupling between the electrons removes the \((2S + 1)\)-fold degeneracy of the ground state. The Hamiltonian for this field-independent interaction is given as

\[ \hat{\mathcal{H}}_{ZFS} = S^T D S \]  

(2.11)

with the symmetric and traceless zero-field splitting tensor given by \( D \). With the diagonalized \( D \) tensor in the principal axis system (PAS), the Hamiltonian can be written as,

\[ \hat{\mathcal{H}}_{ZFS} = D_x \hat{S}_x^2 + D_y \hat{S}_y^2 + D_z \hat{S}_z^2 \]  

(2.12)

\[ \hat{\mathcal{H}}_{ZFS} = D \left[ S_z^2 - \frac{1}{3} S(S + 1) \right] + E (S_x^2 - S_y^2) \]  

(2.13)

with \( D = \frac{3}{2} D_z \) and \( E = \frac{D_x - D_y}{2} \). When the ZFS is much larger than the EZI, the microwave frequency may be too small to excite all the transitions and only a part of the transitions are observed. Electrostatic interaction in a crystal field can lift the spin degeneracy of the unpaired electrons. For systems containing an odd number of group spins, according to Kramers’s degeneracy at least one of the lowest energy levels remains degenerate and will further split in presence of an external magnetic field. Such a Kramer’s doublet can be described with an effective spin \( S = 1/2 \) as in the case of Dy\(^{3+}\). For example, in the case of Gd\(^{3+}\) ions with a spin \( S = 7/2 \), all the transitions have to be taken into account as the ZFS
is comparatively small. The spectrum is rather broad due to a distribution of D and E values and has a pronounced peak corresponding to the central transition.

### 2.1.5 Spin-spin interaction

When two electron spins weakly interact, they can still be described by their individual spins $S_1$ and $S_2$ and their interaction is described by a spin-spin interaction term,

$$\hat{H}_{ee} = S_1 D_{dd} S_2 + J S_1 S_2$$  \hspace{1cm} (2.14)

where $D_{dd}$ is the dipole-dipole tensor and $J$ is the exchange coupling. The Heisenberg exchange coupling term is relevant if the orbitals of the two spins in the system can significantly overlap. In solids, this is the case for interspin distances $< 1$ nm or for strongly delocalized unpaired electrons. If the two radicals are freely diffusing in solution and can collide, this overlap may be transient. As the nitroxide labels are attached via saturated tethers, $J$ coupling is at least one order smaller than dipole-dipole coupling for distances longer than 1.5 nm, which is the case encountered in this thesis. In addition, the frozen buffer or lipid environment with weak conductance reduce $J$ coupling by another order of magnitude. Such small $J$ coupling can be neglected. With the assumptions of the localization of the electron spin in the center of N-O bond in nitroxide radicals and a parallel orientation of the quantization axis along the external magnetic field, the electron-electron dipolar tensor can be described by a point-dipole approximation. The Hamiltonian can be written as a sum of secular and pseudo-secular terms respectively as given below

$$\hat{H}_{ee,sec} = [J + \omega_{dd}(1 - 3\cos^2\theta)]S_{1x}S_{2x}$$  \hspace{1cm} (2.15)

$$\hat{H}_{ee,psec} = [J - \frac{1}{2}\omega_{dd}(1 - 3\cos^2\theta)](S_{1x}S_{2x} + S_{1y}S_{2y})$$  \hspace{1cm} (2.16)

with $\theta$ being the angle between the spin-to-spin vector and the magnetic field axis and $\omega_{dd}$ the dipolar frequency given as,

$$\omega_{dd} = \frac{1}{r_{12}^3 \mu_0 g_1 g_2 \mu_B^2}$$  \hspace{1cm} (2.17)

With an isotropic g value of $g_1 = g_2 = 2.006$ approximated for nitroxide spins, the dipolar coupling has a value of $2\pi \times 52.04$ MHz nm$^{-3}$. In the high field approximation, the pseudo secular term in equation 2.16, which account for the mixing of $|\alpha_1\beta_2\rangle$ and $|\beta_1\alpha_2\rangle$ states can be neglected as the difference of resonance frequencies for the coupled spins is typically much larger than $\omega_{dd}$.

### 2.2 Site-directed spin labeling technique

Since its first demonstration with bacteriorhodopsin [68, 69], site-directed spin labeling
(SDSL) combined with EPR has been applied on a large number of proteins to obtain structural information [70]. EPR spectroscopy requires stable unpaired electrons and for proteins this is achieved by introduction of a spin label via cysteine mutagenesis at a desired site. Thus, the natural cysteine(s) must be removed to create a ‘cys-less’ mutant of the protein for specific incorporation of the spin label. Other than for proteins, SDSL-EPR has been used with other biomacromolecules such as with DNA and RNA to obtain information on structure and dynamics [71, 72].

**Figure 2.3:** Scheme of the spin labeling reaction with MTSSL. The methanethiosulfonate spin label reacts with the free sulfhydryl group of the cysteine. The label is attached via disulfide linkage giving rise to the unnatural side chain R1 carrying an unpaired electron at a desired site. For an effective reaction, no reducing agents should be present during or after spin labeling of the protein.

For spin labeling of proteins, an (1-Oxyl-2,2,5,5-tetramethyl-D3-pyrroline-3-methyl) moiety in the nitroxide-based methanethiosulfonate spin label (MTSSL, referred as NO) is the most widely used (figure 2.3). Its relatively small size, similar to that of a tryptophan side chain, its high specificity for the free thiol of cysteines and its flexibility make MTSSL the preferred spin-label in proteins’ studies. On the other hand, the label flexibility causes the variation of Cα-NO distance in the 4-8 Å range depending on the position, making it difficult to directly relate the obtained NO-NO distance data to the coordinate of the native side chain. MTSSL is attached via disulfide linkage and as such it is susceptible to cleavage under reducing conditions. Under slightly reducing conditions, maleimido (MSL) or iodoacetamido (IASL) spin labels can be used.

**Figure 2.4:** Gadolinium(III)-based labeling of cysteines. As an example the maleimido mono amide DTPA chelating agent for Gd(III) which reacts with the free sulphydryl group of the cysteine is shown. In this case reducing agents can be present during or after spin labeling of the protein.
In the case the naturally occurring cysteines may not be removed from a protein, specific labeling with a hydroxylamine spin label of a genetically encoded unnatural amino acid (p-acetyl-L-phenylalanine) has been also demonstrated with T4 lysozyme [73]. Though this is a potential approach for specific labeling of proteins containing several natural cysteines, requirement of an acidic pH (4.0) for labeling somewhat limit its general application. Recently gadolinium(III)-based labels (figure 2.4) were introduced [90-91], which can be used in SDSL-EPR orthogonally to the conventional nitroxide-based labels [74-79]. Their application on peptides and water soluble proteins were demonstrated. In this thesis the first application of this new type of labels to membrane proteins will be described.

2.3 The nitroxide spectrum: continuous wave EPR

Continuous wave (cw) EPR spectroscopy of the nitroxide spin labels attached to biomacromolecules can provide information on solvent accessibility, polarity, side chain mobility and distance between the spin labels [80]. These parameters obtained for several positions in a protein can be used to determine secondary structure and its orientation and protein topography. For nitroxide radicals, the unpaired electron is partially located in the 2p_z-orbital of nitrogen. In the molecular coordinate system for nitroxide, this orbital is along the z-axis and the N-O bond is along the x-axis and the y-axis is perpendicular to both x- and y-axis (Fig. 2.5).

![Figure 2.5: Energy level splitting for nitroxide radicals and powder spectra at X band. Left) Zeeman and hyperfine splitting scheme for a ^{14}N nitroxide radical (S=1/2, I=1). Middle) Structure of a pyrroline-type nitroxide. The coinciding g and A tensor frames are shown relatively to the p_z orbital of the nitrogen atom. Right) Due to the anisotropy of the hyperfine interaction with the ^{14}N nucleus, different orientations with respect to the magnetic field lead to different hyperfine splitting. Separate effects of the hyperfine and g anisotropy on each m_I line computed numerically. At the bottom absorbance and first derivative “powder spectrum” at X band. Adapted from http://www.epr.ethz.ch/education/epr2007.pdf](http://www.epr.ethz.ch/education/epr2007.pdf)
The typical continuous wave EPR spectrum of a nitroxide radical consists of three hyperfine lines due to the hyperfine interaction with the nuclear spin of the most abundant $^{14}$N nucleus ($I=1$). The hyperfine splitting of a nitroxide spectrum depends on the relative orientation of the nitroxide molecular frame with respect to the external magnetic field $B_0$ (figure 2.5).

The orientation-dependent properties of the nitroxide spectrum are determined by the anisotropy of the A and g tensors. For MTSSL, the hyperfine values are $A_{xx} \approx A_{yy} = 0.45-0.50$ mT and $A_{zz} = 3.3-3.7$ mT (polarity dependent) and the g values are $g_{xx} = 2.0081-2.0091$ (polarity and proticity dependent), $g_{yy} = 2.0061$ and $g_{zz} = 2.0021$. The typical room temperature continuous wave EPR spectrum of a freely tumbling MTSSL in water consists of three equally spaced narrow lines centered at an average g value due to fast averaging of the anisotropies. In the rigid limit, e.g. at low temperature, the weighted superpositions of all possible molecular orientations leads to the so called “powder spectrum” characterized at X band by a central $m_I = 0$ line with a frequency dispersion solely caused by g anisotropy and by a high field line broader than the low field line due to the relative sign of the A and g values (figure 2.5).

![Figure 2.6: Effects of molecular motion and different frequencies on the nitroxide spectrum. A) Simulations of powder spectra performed with the Easyspin function ‘pepper’ at X (9.5 GHz, 0.34 T), Q (34 GHz, 1.2 T), and W (95 GHz, 3.4 T) bands. The g and A parameters resolved in the W-band spectra are shown. B) Simulations of nitroxide spectra in the intermediate motional regime performed with ‘chili’ for isotropic reorientational motion with rotational correlation time of 1 ns. C) Nitroxide spectra in the fast motional regime simulated with ‘garlic’ for isotropic reorientational motion with rotational correlation time of 10 ps.](image)

EPR spectra are sensitive to molecular motions. The motion of nitroxide spin labels attached to a protein molecule is influenced by the rotational correlation time of the entire protein, the rotational isomerization around the bonds attaching the spin label to the protein and motion of the backbone segment relative to the entire protein. At X band the spectrum of a nitroxide is dominated by the hyperfine splitting. If the spin labels rotates with an effective rate exceeding the anisotropy of the hyperfine interaction (fast motion regime), a simple triplet spectrum characterized by an isotropic hyperfine coupling is
2.3 Nitroxide spectrum

observed (figure 2.6). In the rigid limit, the spectra consist of a superposition of all the possible orientations with the maximum hyperfine coupling of $2A_{zz}$ between the outer peaks (figure 2.6A). In between these limits, a range of intermediate linewidths can be detected (figure 2.6B).

For spin labels attached to proteins, a range of different anisotropic reorientational motions can occur depending on the site of labeling, giving rise to a plethora of different spectra characterized by different “mobilities”, which are encoded in the EPR lineshape at room temperature, and can be used to extract valuable information about secondary and tertiary structures of proteins, and about their conformational transitions. As an example in figure 2.7 the two spectra of spin labels attached to the ABC importer BtuCD in the cytoplasmic and periplasmic gate are presented, highlighting the different mobilities of the two spin-labeled sites.

In the case two spin labels in a protein are characterized with an interspin distance < 2 nm, the distance information can be extracted from the dipolar broadening visible directly in the cw-EPR spectrum at low temperatures. The software DIPFIT developed by Steinhoff et. al can extract distances from the dipolar broadening [81]. DIPFIT needs spectra in the “rigid limit” measured at cryogenic temperatures (< 200 K) to suppress the residual motion of the spin labels and the dipolar averaging.

**Figure 2.7:** X band room temperature spectra of nitroxide labels attached to BtuCD. Left) Crystal structure of BtuCD (PDB code 1L7V). The R1 side chains attached at the cytoplasmic and periplasmic gates (positions 141 and 168, respectively) are calculated and visualized using the MMM software approach. Two spectral fractions associated with a mobile (m) and more immobile (i) component at position 168 are highlighted with arrows.

The program simulates and convolutes a non-interacting spectrum and fits the dipolar-broadened spectra with a Gaussian distance distribution for distance $r$ with a standard deviation ($\sigma_r$). A high spin labeling efficiency for the protein sample is required by DIPFIT for precise calculation of distance. For distances shorter than 1.4 nm, the accuracy of the calculated distances decreases, as the exchange coupling is not included in the fitting. Similarly, for distances longer than 2 nm, the accuracy gradually decreases, as the dipolar broadening is indistinguishable from the natural line width.
2.4 Double Electron Electron Resonance (DEER)

When the interspin distance is > 2 nm, pulse EPR techniques are required to extract the dipolar coupling (thus the interspin distance) between two unpaired electrons. Double electron-electron resonance (DEER) also called pulsed electron double resonance (PELDOR) can measure the weak dipolar coupling between unpaired electrons spatially separated by 2-10 nm [70]. The technique involving a three-pulse sequence was first implemented by Milov et al. [82]. Later, a four-pulse sequence (figure 2.8) for dead time free detection of the signal was developed [83, 84] and nowadays it is widely used with nitroxide labels to study structure and dynamics of biomacromolecules [70].

The DEER experiment contains a refocused echo sequence for one type of spins (observer spins) whose echo amplitude is modulated as a function of the timing of the excitation of another type of spins (pump spins), which are coupled to the observer spins. The modulation of the echo arises from changing the local magnetic field exerted by the pumped spins on the observer spins through dipolar interaction (figure 2.8).

**Figure 2.8:** Dead time-free four-pulse DEER. **A)** Pulse sequence for the double frequency experiment. **A)** A refocused echo is created by a sequence (blue) of one \( \pi/2 \) and two \( \pi \) pulses at frequency \( v_{\text{obs}} \) and a \( \pi \) pulse at frequency \( v_{\text{pump}} \) (red) is applied at different time intervals during the experiment. **B)** The \( \pi \) pulse at frequency \( v_{\text{pump}} \) inverts the pumped spins (red), thus changing the local field at the observer spins (blue). **C)** Echo-detected field swept spectrum of a nitroxide radical at X- and Q-band frequencies. The positions of the observer and pump pulses are depicted. At X band, the pump and observer frequencies are separated by +65 MHz and at Q band, the frequency separation is -100 MHz. **D)** DEER data analysis. The primary DEER data \( V(t) \) fitted with an exponentially decaying background (orange line in left panel). The form factor \( F(t) \) is obtained by dividing the primary data by the background, subtracting one (middle). It oscillates with a characteristic frequency determined by the interspin distance. The inversion efficiency \( \lambda \) depends on the inversion efficiency of the pump pulse and on the labeling efficiency. The fitting of the form factor with simulated time domain data \( S(t) \) and Tikhonov regularization gives the interspin distance distribution.
The echo detected field sweep spectra of a nitroxide radical at X- or Q-band frequencies are very broad and it is possible to selectively excite a fraction of the spins. Thus two different frequencies are employed in DEER; one frequency for the pulse sequence to create a refocused echo of the observer spins and the second frequency to invert the pumped spins, separated by +65 or -100 MHz at X- or Q-band respectively. The pulse sequence of the refocused echo is invariant and the timing of the pump pulse is changed during the experiment.

The resulting signal is composed of two parts: an exponentially or stretched exponentially decaying background function \( B(t) \) due to intermolecular interactions of unpaired spins in different protein molecules or protein complexes and a form factor \( F(t) \) due to interactions of the coupled spins within the same protein or protein complex.

\[
V(t) = B(t) \times F(t)
\]

With \( V(0) = 1 \). By assuming an exponential decay for the background function that depends on the dimensionality of the distribution of the spin labels, \( B(t) \) is given by

\[
B(t) = \exp(-kt^D)
\]

where \( k \), quantifies the density of the spins. Proteins in frozen glassy solutions are homogenously distributed in three dimensions \( D = 3 \). Membrane proteins reconstituted into liposomes may be confined to a two-dimensional bilayer and a value for \( D = 2 \) may be assumed. It is possible to experimentally determine the value of \( D \) for doubly-labeled proteins using the corresponding singly-labeled species. Such experimental determination is difficult with dimers or oligomers. It possible to extract the form factor by dividing the primary DEER data with the background function. For a given angle \( \theta \) between the interspin vector and the external magnetic field, the form factor is a function of the dipolar frequency \( \omega_{dd} \) (equation 2.17), of the modulation depth \( \lambda \), which is determined by the inversion efficiency of the pump pulse and of the labeling efficiency.

\[
F(t) = 1 - \lambda[1 - \cos((1 - 3\cos^2\theta)\omega_{dd}t)]
\]

According to equation 2.17, long distances correspond to low dipolar frequencies \( \omega_{dd} \), whose precise measurement requires long observation times. Interspin distances between spin labels incorporated on a frozen protein sample are characterized by a broad distribution due to the flexibility of the labels and possibly due to the flexibility of the protein backbone. Thus the form factor encodes not only the distance, but the distribution of the distances as well. Assuming an isotropic distribution of the interspin vector along the external magnetic field, the form factor component for each distance can be transformed into a Pake pattern using Fourier transformation. Thus the experimental \( F(t) \) is fitted by a
linear combination of multiple traces each corresponding to a single distance. The coefficients in this linear combination of multiple traces constitute the distance distribution.

2.4.1 Sensitivity enhancement of DEER experiment

The transverse relaxation time $T_2$ restricts the upper limit for the total length of the DEER sequence, and thus the upper limit for the distance measurements in DEER experiments. Following a $\pi/2$ pulse, the phase coherence is lost over time, mostly due to spin flip-flop process and magnetic field inhomogeneity. The loss of coherence due to field inhomogeneity can be reversed by a refocusing $\pi$ pulse and by measuring the echo intensity as a function of inter-pulse delay ($\tau$), the phase memory time $T_m$, which is approximately equal to $T_2$ can be calculated.

The signal-to-noise ratio of the DEER data does not improve proportionally to the spin concentration as instantaneous diffusion leads to loss of phase memory at high spin concentrations [72]. This phenomenon is particularly important when long distances are measured, where the total length of the pulse sequence exceeds the phase memory time. For reliable extraction of a distance $r$, the dipolar evolution needs to be measured to a time $t_{\text{max}}$ given by

$$t_{\text{max}} \geq \frac{8\pi \hbar T_{12}^2}{g_1 g_2 \mu_B^2}$$  \hspace{1cm} (2.21)

With the approximation that the total time for the electron coherence evolution $t_{\text{total}} \approx 2t_{\text{max}}$, the optimal concentration for a DEER experiment to measure a distance $r$ is given by [72]

$$C_{\text{opt}} = \frac{g \sqrt{3}}{2\pi} \frac{h}{t_{\text{max}} f_{1,\pi} N_1 g_1 g_2 \mu_B^2}$$  \hspace{1cm} (2.22)

where $f_{1,\pi}$ is the fraction of observer spin excited by the observer $\pi$ pulse and $N_1$ Avogadro’s constant. By replacing $t_{\text{max}}$ with equation 2.21, a plot of optimum concentration as a function of distance can be obtained (figure 2.9).

Thus the measurement of long distances (> 6 nm) with DEER suffers from requirement of low spin concentration and evolution time longer than the phase memory time. The signal-to-noise-ratio in general for DEER data depends on the temperature of the measurement as well. The longitudinal relaxation time $T_1$, the transverse relaxation time $T_2$ and the polarization of the spin transition from Boltzmann distribution are temperature dependent. For nitroxide spin labels in solids, $T_2$ increase with decreasing temperature until an asymptotic value, whereas $T_1$, which determines the maximum repetition rate of the experiment, increases monotonically. $T_2$ can be further increased by matrix deuteration [85]. It has been shown that a measurement temperature of 50 K is close to the optimum, independent of the nature of materials carrying the nitroxide radicals and the frequency of
2.4 Double Electron Electron Resonance

Figure 2.9: Relation between optimum concentration of nitroxide spin labels and the expected mean distance. The solid line corresponds to equation (2.22), the dotted line to a maximum dipolar evolution time $t_{\text{max}}$ necessary to obtain a reliable shape of a narrow distance distribution \[72\].

the measurement \[72\]. As for membrane reconstituted proteins, $T_m$ is significantly reduced in the lipid environment, which limits the extent of measurable distances. At this temperature $T_m$ in the order of 1-2 $\mu$s and $T_1$ in the order of 1-2 ms can be achieved for spin-labeled membrane proteins in the presence of deuterated glycerol as cryoprotectant.

The sensitivity of the DEER experiments can be further improved with pulses having optimum length. In general the sensitivity increases with decreasing the pulse lengths for the given flip angles. Short pulses require a high microwave field strength and for a given resonator this is limited by the available microwave power in the resonator. By combining an improved resonator with short pulses (all 12 ns) enabled by a high-power amplifier at Q-band frequency, it has been shown that the sensitivity of the DEER measurement could be significantly improved \[86\].

2.4.2 Data analysis

The fitting of the primary DEER data to the form factor provide the distance distribution $P(r)$. Such fitting using Tikhonov regularization with L-curve criterion is implemented into the MATLAB-based software package DeerAnalysis \[87\]. The computation of distance distribution $P(r)$ from the dipolar evolution function $F(t)$ is an ill-posed problem \[72\]. The reliability of the distance distributions strongly depends on the signal-to-noise ratio of the data. Therefore the fitting aims at improved resolution in the distance distribution while decreasing the influence of experimental noise. The distance distribution is characterized by three important features, the shape, the mean distance $<r>$ and the width of the distance distribution $\sigma_r$. To reliably extract all the three parameters, the dipolar evolution needs to be measured at least two periods of the dipolar oscillation for the mean distance and the background needs to fitted at times longer than one period. DeerAnalysis computes a time-domain signal $S(t)$ for a given distance distribution $P(r)$ using a kernel function and tries to fit it to the experimental form factor $F(t)$ with minimum deviation.

$$S(t) = K(t,r)P(r)$$ \hspace{0.5cm} (2.23)

The kernel function $K$ for DEER experiments employing ideal pulses is given by
Chapter 2. EPR spectroscopy

\[ K(t, r) = \int_0^1 \cos[(3x^2 - 1)\omega_{dd}t]dx \]  

(2.24)

Because the problem is ill-posed, straight fitting of \( S(t) \) to \( F(t) \) would result in strong noise artifacts. DeerAnalysis employs Tikhonov regularization to deal with this ill-posedness of the problem. A regularization parameter \( \alpha \) is used to find the optimum distance distribution by minimizing the function,

\[ G_\alpha(P) = \|S(t) - D(t)\|^2 + \alpha \left\| \frac{d^2}{dr^2} P(r) \right\|^2 \]  

(2.25)

with \( D(t) \) given by \( \frac{F(t) - (1-\lambda)}{\lambda} \).

The first term minimizes the error in fitting of the simulated dipolar evolution function \( S(t) \) to the experimental data \( D(t) \). The second term gives the square norm of the second derivative of \( P(r) \) weighted by the regularization parameter \( \alpha \), which is a measure of the roughness of \( P(r) \). Large \( \alpha \) means a broad distance distribution and for well-defined narrow distances a smaller \( \alpha \) is required.

![Figure 2.10: Tikhonov regularization L curve. Left) Plot showing the relation between mean square deviations \( \rho \) and smoothness of the distribution \( \eta \) (2.25-2.27) with different values of \( \alpha \) highlighted. Right) Distance distributions obtained with different values of \( \alpha \) under the constraint \( P(r) > 0 \). Red, undersmoothed distribution with unrealistic peak splitting; blue, oversmoothed distribution with artificial broadening; green, distance distribution corresponding to \( \alpha = 1 \), which is most realistic in this case [70].](image)

The optimum \( \alpha \) is calculated by the L curve criterion (figure 2.10), which is a parametric plot of \( \log \eta(\alpha) \) versus \( \log \rho(\alpha) \) defining the mean square deviation and the smoothness respectively, defined as

\[ \rho(\alpha) = \|S(t) - D(t)\|^2 \]  

(2.26)
2.5 DEER employing Gd(III)-NO pairs

\[ \eta(\alpha) = \left\| \frac{d^2}{d\tau^2} P(r) \right\|^2 \]

For data with good signal-to-noise this plot is L-shaped (figure 2.10). The green point in the curve defines the optimum value for the regularization parameter \( \alpha = 1 \).

2.5 DEER employing Gd(III)-NO spin pairs

Traditionally, DEER is most frequently used on two nitrooxide radicals (mostly MTSSL) incorporated at desired sites in a protein. The combination of nitrooxide radicals with Cu(II) metal center in a porphyrin model complex for DEER measurement has been demonstrated [88]. Distance measurement using Cu(II)-Cu(II) and Gd(III)-Gd(III) spin pairs was performed using DEER [89, 90]. Gd(III) probes are promising for high field DEER applications as the orientation selection is much less problematic due to stochastic distribution of the eigenframe orientations and magnitudes of zero-field splitting [91].

![Figure 2.11: Gd(III)-NO DEER measurements. A) Field swept echo detected spectra of [Gd(DOTA)] (black) and nitroxide radicals (red) at different experimental conditions in terms of power and shot repetition time. The difference in microwave power setting between nitroxide and Gd(III) is 12 dB. Shot repetition times of 100 ms for the nitroxide and 1 ms for Gd(III) were employed. Arrows mark positions of pump and detection pulses. Spectra were normalized to their maximum intensity. B) Corresponding spectra in a broader field range. C) DEER sequence for Gd(III)-nitroxide distances. Standard settings (as well as settings with smaller flip angles \( q \) for the pumping pulse were applied. D) Ribbon model of wild-type T4-lysozyme with the spin labeled sites. Adapted from Garbuio et al. 2013.](image)

Orthogonal labeling using Gd(III) and NO spin labels was used for determining selective Gd(III)-NO and NO-NO distance measurements on model systems and soluble proteins [74-78]. The DEER setup is briefly introduced in figure 2.11. It has been shown that for Gd(III)-NO DEER at X- and Q-band frequencies, high-field approximations are valid and the
analysis routines employed in the DeerAnalysis package can be used for data analysis [74]. In this thesis I demonstrate for the first time the application of site-directed orthogonal spin labeling and selective distance measurements using Gd(III) and NO probes on a membrane protein. This strategy is used to investigate simultaneously the BtuCD-BtuF interaction (via Gd(III)-NO distance) and gate conformation (via NO-NO distance) using liposome reconstituted samples.
This work was published (Joseph et al. 2011) with Birke Goetz, Kaspar P Locher, Gunnar Jeschke and Enrica Bordignon as the co-authors. All the biochemical and EPR works were done by me. Birke Goetz helped with establishing BtuCD purification.

3. Transmembrane gate movements in the type II ABC importer BtuCD-F during nucleotide cycle

3.1 Introduction

ABC transporters couple the energy of ATP hydrolysis to the translocation of substrates across biological membranes. They constitute the largest transmembrane protein family present in all branches of life and mediate the active transport of various substances like sugars, amino acids, peptides, vitamins, iron siderophores, opines, metals, etc. across the membrane [1, 45, 92]. Biochemical evidence supports a ‘two-state, alternating-access’ mechanistic model for both ABC exporters and importers [38]. In this model, an ATP-bound conformation of the transmembrane domains facing the extracellular side of the membrane is converted to an inward-facing conformation via ATP hydrolysis in the nucleotide binding domains (NBDs). This conformational transition ensures net substrate uptake by the importers or net expulsion by the exporters. Among the canonical ABC importers, which are characterized by the presence of a soluble periplasmic substrate binding protein, two structurally different types exist, namely type I (e.g. maltose, molybdate, methionine systems) and type II (e.g. vitamin B₁₂, heme, metal systems) [45]. Type I are rather well characterized ABC importers, for which the details of the alternating-access mechanism have been confirmed by a large body of experimental evidence, including the crystal structures showing the maltose transporter in different states during the nucleotide cycle [28, 35, 37, 93]. The transmembrane domains (TMDs) of type I importers, featuring 10-14 helices, alternate from an ATP-bound outward-facing conformation where the substrate binding protein releases its cargo to the low affinity binding site in the TMDs, to an ADP-bound inward-facing conformation where the substrate is released in the cytoplasm. Type II importers comprise up to 20 TM helices, which can translocate substrates up to one order of magnitude bigger than those imported by the type I (e.g. vitamin B₁₂ - 1355 Da; methionine - 141 Da). Up to date, only two type II importers have been crystallized: the BtuCD vitamin B₁₂ importer from E. coli in the presence and absence of the substrate binding protein BtuF [8, 15] and the BtuCD homologous putative metal-chelate- importer HI1470/1 from H. influenzae [16]. The BtuCD structure first obtained with the translocation pathway open to periplasm in a nucleotide-
free state suggested that this transporter might operate by a mechanism opposite to that of type I transporters. However, the homologous Hi1470/1 importer was later crystallized in a nucleotide-free inward-facing conformation, similar to the apo-state of the type I importers. The structure of BtuCD-F with an occluded asymmetric translocation pathway suggested further mechanistic diversity between type II and type I importers. Overall, the three crystallized states confirm the possibility of an alternating-access mechanism for substrate transport. However, unlike the maltose transporter, the absence of nucleotides in all the three structures makes it impossible to delineate the sequence of the conformational switches during the transport cycle.

Besides the structural diversity between the two types of importers, remarkable differences exist in the interactions between the substrate binding proteins and the TMDs. The affinity of the substrate binding protein is orders of magnitude larger in type II importers ($K_d \approx 10^{-13}$ and $10^{-9}$ M, for BtuCD-F and Hi1470/1-2, respectively) [58] than in type I ($K_d \approx 10^{-4}$ M) [94]. Additionally, no substrate binding site could be identified in the type II TMDs. Another striking difference is that the BtuF affinity for BtuCD is decreased by 2 orders of magnitude in the presence of ATP (and in the transition state intermediate) [58]. The affinity is further reduced by high concentrations of substrate. By contrast, the highest affinity of the substrate binding protein to type I importers is in the transition state intermediate [95] and no evidence for substrate-induced changes in affinity was found. All these observations highlight the mechanistic differences between type I and type II importers.

Probe techniques, such as site-directed spin labeling [96] allow for studying conformational transitions during the transport cycle in ABC transporters [97-102]. Methanethiosulfonate spin labels (MTSSL) were previously attached in BtuCD to cysteines strategically placed at the cytoplasmic end of TM5 (Ser141) and in the short periplasmic loop between TM5 and TM5a (Thr 168) [8, 101], which are key residues in the substrate translocation channel (figure 3.1). It was shown by electron paramagnetic resonance (EPR) that in the absence of BtuF the dynamics of the two gates were unaffected by the presence of nucleotides. In contrast, the presence of BtuF induced changes in the dynamics of both gates, indicating a downstream communication from the periplasmic to the cytoplasmic region of the transporter mediated by BtuF. Addition of AMPPNP (a non-hydrolyzable analogue of ATP) switched the cytoplasmic gate to a highly mobile conformation [16]. Although an increase in mobility is not generally correlated with an increase in distance between the two spin labels, the authors of (11) proposed that the higher mobility in the AMPPNP-bound BtuCD-F may reflect an inward-facing conformation.

In this work we used spin-labeled cysteines preceding and following TM5 (residues Ser141, Thr142 and Thr168, respectively) to quantitatively follow the movement of the cytoplasmic and periplasmic gates during the nucleotide cycle using pulse EPR techniques. In the present report we delineate the sequence and molecular details of the opening and closing of the translocation pathway gates during the nucleotide cycle. The
combined effects of substrate and nucleotides on the affinity of BtuF for the TMDs are also described. We could conclusively observe that the cytoplasmic gate of BtuCD-F opens upon ATP binding which could facilitate the release of vitamin B\textsubscript{12} into the cytoplasm. An EPR-based model of a productive translocation event is provided.

### 3.2 Experimental procedures

#### 3.2.1 Expression and Purification of BtuF and BtuCD

BtuF was purified as described before [103]. BtuF loaded with vitamin B\textsubscript{12} was stored at 4°C up to three months. Protein concentration was determined by absorption at 361 nm. Cysteine mutations in BtuCD at positions 141, 142 and 168 were introduced on a “cys-less” plasmid as described before [8]. BtuCD was extracted and purified in LDAO (lauryldimethylamine-N-oxide) as described earlier [15] with some modifications. BtuCD mutants were overexpressed in *E. coli* BL-21-(DE3) Gold cells. The frozen cells were solubilized in 1% LDAO (Anatrace) in 50 mM Tris-HCl (pH 7.5) containing 25 mM imidazole-HCl (pH 8.0), 5 mM β-mercaptoethanol and 500 mM NaCl. The cells were lysed by sonication using a Sonics vibra-Cell sonicator. The lysate was centrifuged for 30 minutes at 40,000 x g and then loaded onto a Ni-NTA column, pre-washed with 50 mM Tris-HCl (pH 7.5) containing 0.1% LDAO, 25 mM imidazole-HCl (pH 8.0), 5 mM β-mercaptoethanol and 500 mM NaCl. The column was washed with 50 mM Tris-HCl (pH 7.5) containing 0.1% LDAO, 90 mM imidazole-HCl (pH 8.0) and 500 mM NaCl. BtuCD was eluted with 200 mM imidazole in 50 mM Tris-HCl (pH 7.5) containing 0.1% LDAO and 500 mM NaCl. The protein was immediately desalted with a HiPrep desalting column (GE Healthcare) into 50 mM Tris-HCl (pH 7.5) containing 0.1% LDAO and 500 mM NaCl. Protein aggregation was checked by size-exclusion chromatography with a Superdex 200 10/300 column (GE Healthcare).

#### 3.2.2 Spin labeling of BtuCD mutants

After desalting, BtuCD was concentrated to 15-20 µM with 50 KD Amicon Ultra Concentrators (Millipore). A 40 fold molar excess of MTSSL ([1-oxyl-2,2,5,5,-tetramethyl-d-3-pyrrrole-3-methyl) methanethiosulfonate] was added in 4 batches every 5 min at room temperature while shaking. To prepare BtuCD-F, vitamin B\textsubscript{12}-bound BtuF was added at this stage at a molar ratio of 1:2 BtuCD:BtuF. Excess MTSSL was removed using PD10 desalting columns (GE Healthcare). The functionality of protein preparations was analyzed with ATPase assay as described [103]. For experiments in LDAO, the protein was concentrated to 100 µM using Amicon Ultra Concentrators (Millipore). At this stage samples could be snap-frozen in liquid nitrogen in small aliquots and stored at -80°C for further use up to three months.
3.2.3 Reconstitution of BtuCD and BtuCD-F in liposomes

For reconstitution BtuCD and BtuCD-F were concentrated to 30 µM and reconstitution was performed according to the protocol described before by exchanging the LDAO to Triton X-100 [103] or directly from LDAO supplementing the sample with 0.14% Triton X-100 and incubating it with liposomes for 1 h at room temperature. Liposomes were pre-incubated with 0.14% Triton X-100 for 1 h. A 1:500 molar ratio of BtuCD to lipids was used. Reconstitution was performed using BioBeads SM-2 as described [103]. ATPase activities were measured for all spin-labeled mutants according to Borths et al. [103].

3.2.4 Sample preparation for EPR measurements

BtuCD and BtuCD-F in LDAO or in liposomes were prepared in the apo-state (absence of nucleotides). The ATP-bound state was prepared incubating the sample for 5 minutes at 4°C with 1 mM ATP, 50 µM EDTA. The ATP analog AMPPMP was also used at 1 mM concentration. The post hydrolytic state was induced by incubation of the sample for 10 minutes at 37°C with 1 mM ATP and 2 mM MgCl₂ or directly by incubation at 4°C for 5 minutes with 1 mM ADP and 2 mM MgCl₂. For DEER (Double Electron Electron Resonance) measurements, 10% v/v deuterated glycerol was added to the samples before snap freezing them in liquid nitrogen.

3.2.5 Continuous wave and pulse EPR

All continuous wave (cw) X-band EPR experiments were performed with a Bruker Elexsys E580 spectrometer equipped with a Bruker Elexsys Super High Sensitive probehead at room temperature or at 160 K using a Bruker N₂ flow cryostat. EPR spectra were detected at room temperature in EPR glass capillaries (0.9 mm inner diameter, sample volume 15 µl) with 100 kHz field modulation, 2 mW microwave power, 0.15 mT modulation amplitude. EPR spectra for interspin distance determination were recorded at 160 K in EPR quartz capillaries (3 mm outer diameter, sample volume 30 µl) and recorded with 100 kHz field modulation, 0.08 mW microwave power, 0.25 mT modulation amplitude. Fitting of dipolar broadened EPR powder spectra was performed with the software DIPFIT [81]. The linewidth parameters of the reference non-dipolar-broadened spectrum for each spin-labeled position in LDAO or in liposomes were obtained by fitting the spectrum in the nucleotide state which showed distances >2 nm in DEER. The dipolar broadened spectra were fitted by fixing all parameters, except Azz, distance and distance distribution (Gaussian model).

DEER measurements were performed at X-band frequency with a Bruker Elexsys E580 spectrometer equipped with a Bruker Flexline split-ring resonator ER 4118X-MS3 using a continuous flow He cryostat (ESR900; Oxford Instruments) controlled by an Oxford Instruments temperature controller ITC 503S. DEER measurements were also performed
3.2 Experimental procedures

at Q-band frequency (34-35 GHz) on a home-made spectrometer equipped with a home-made rectangular resonator enabling the insertion of X-band sample tubes with outer diameter 3 mm [104]. Dipolar time evolution data were acquired using the four-pulse DEER experiment. All DEER measurements were performed at 50 K. For X-band DEER, observer pulse lengths were set to 32 ns for $\pi/2$ and $\pi$ pulses, with the ELDOR $\pi$ pulse set to 12 ns. The ELDOR frequency was set at the maximum of the echo-detected field swept spectrum, 65 MHz lower than the observer frequency. Deuterium nuclear modulations were averaged by increasing the first interpulse delay by 56 ns for 8 steps. For Q-band DEER, all pulses were set to 12 ns and deuterium nuclear modulations were averaged by increasing the first interpulse delay by 16 ns for 8 steps. The ELDOR frequency was set at the maximum of the echo-detected field swept spectrum, 80 MHz higher than the observed frequency. Traces were accumulated for 2-6 h at Q band and for 24-48 h at X band depending on spin concentration. The background of the DEER primary data ($V(t)$) was fitted and the resulting secondary data ($F(t)$) were converted by a model-free Tikhonov regularization to distance distributions with the software DeerAnalysis2010 [87]. The simulation of the possible spin label rotamers attached at a position was performed using the Matlab program package MMM (http://www.epr.ethz.ch/software/index) based on a rotamer library approach [105].

3.3 Results

The crystal structures of BtuCD-F suggested that the periplasmic (T168C) and the cytoplasmic (S141C, T142C) gate residues offer key positions to follow conformational changes during the transport cycle [8, 15]. Figure 1 shows the crystal structures of the vitamin B$_{12}$ importer, with the two gate positions spin labeled in silico with MTSSL based on a rotamer library approach implemented in the software MMM [105]. The simulation provides the spin label rotamers which can be populated in the structures and predicts the distances between the nitroxides of the two labeled sites. Based on the simulations, interspin distance measurements should reveal the effect of BtuF binding, especially at the periplasmic gate.

In the last years DEER (also known as PELDOR, Pulsed Electron Electron Double Resonance) has been widely used as the most sensitive technique to extract distances in the 2-6 nm range [72] on a variety of membrane proteins. For shorter distances (1-2 nm) the analysis must be complemented with lineshape fittings of low temperature continuous wave (cw) EPR spectra [81].

In this work DEER is used to measure interspin distances between the spin-labeled R1 side chains at positions 168, 141 and 142 in BtuCD and BtuCD-F (in the following R1 will denote the unnatural side chain carrying the nitroxide radical). Prior to the EPR analysis, ATPase activities were measured for all spin-labeled mutants both in LDAO and in liposomes. In LDAO the ATPase activities were found to be similar to those measured in
the wild type transporter [103], except for position 142R1, which showed a 50% activity reduction. Nevertheless, the 142R1 mutant in LDAO was proven to be able to bind ATP and ADP, undergoing conformational changes in line with those observed in 141R1. In proteoliposomes all spin-labeled mutants showed wild-type-like ATPase activity, with position 141R1 being the most active (table S1).

![Diagram of BtuCD and BtuCD-F: simulation of interspin distances](image)

**Figure 1.** BtuCD and BtuCD-F: simulation of interspin distances. A) Ribbon representation of the X-ray structures of BtuCD and BtuCD-F (PDB 1L7V and 2QI9, respectively) showing transparent blue ball and stick representation the calculated spin label rotamers attached to the engineered cysteines S141C, T142C and T168C with the software MMM. Color code: yellow - BtuC, green - BtuD, red - BtuF. The TM5 helices containing the strategically placed spin-labeled residues at the cytoplasmic and periplasmic gates (positions 141, 142 and 168, respectively) are highlighted in blue. B) Interspin distances between the gates in BtuCD and BtuCD-F calculated using MMM. Color code: dotted grey - BtuCD, dotted black - BtuCD-F.

### 3.3.1 Opening and closing of the gates in liposome-reconstituted BtuCD-F during nucleotide cycle

The purified BtuCD-F complexes were reconstituted in the presence of vitamin B₁₂ into liposomes. The final proteoliposomes were colorless, indicating absence of vitamin B₁₂, as observed before [8]. Specifically, the sample was measured in three different states: in the absence of nucleotides, in the presence of ATP and EDTA (to prevent hydrolysis), and in the post-hydrolytic state obtained either by incubation with ATP and MgCl₂ or by direct addition of ADP and MgCl₂. The post-hydrolytic states obtained with both methods revealed similar DEER distance distributions in proteoliposomes, although incubation with ATP and MgCl₂ showed some residual distances of the ATP-bound state (figure S7). The
3.3 Results

Dynamics of the R1 side chains in liposomes at positions 141 and 168 as revealed by room temperature cw EPR (figure S1) were found to be in perfect agreement with the previously published spectra [101]. The DEER analysis performed on BtuCD-F reconstituted in liposomes labeled at positions 141, 142 and 168 is presented in figure 2.

It has been shown that the reconstitution method used here leads to 93% of the BtuCD molecules exposing the NBDs to the outside of the liposomes [103]. In line with that, we were unable to see any interaction when BtuF was added to BtuCD reconstituted in liposomes (data not shown). BtuCD-F was also proven by EPR to mainly expose the NBDs to the outside of the liposomes: for all positions investigated an almost complete change of the distance distributions (without residual apo-state distances) was induced upon ATP addition (figure 2). This selective preferential orientation of the transporters in liposomes facilitated data interpretation. The main distance in the apo-state between positions 168 in the periplasmic gate is 2 nm (figure 2A).

**Figure 2**: Nucleotide-dependent movement of periplasmic (A, 168-168) and cytoplasmic (B, 141-141, C, 142-142) gates of BtuCD-F in proteoliposomes. **Left panel)** Normalized experimental data V(t)/V(0) and an exponentially decaying background arising from the distribution of remote spins (dashed lines), as fitted by DeerAnalysis2010. DEER traces were recorded at X band for positions 141 and 168, at Q band for position 142. The background was fitted with an exponential function for position 142 (dimension 3 in DeerAnalysis2010) or stretched exponential function (dimension 3.5, for positions 141 and 168). **Middle panel)** Background corrected normalized form factor F(t)/F(0) and fit by the Tikhonov regularization. **Right panel)** Distance distribution P(r) obtained with...
DeerAnalysis2010. For positions 141 and 168 the excitation bandwidth correction was applied for all traces. The inset shows the spin-normalized cw EPR spectra detected at 160 K. The Gaussian distance distributions obtained by DIPFIT (position 168: ATP-state, red dotted; position 141: apo-state, black dotted, from figure. S2) are superimposed to the DEER-derived distributions in the P(r) panel. Color codes: black, apo; red, ATP; green, ATP and MgCl₂; sea green, ADP and MgCl₂.

ATP binding decreases the interspin distance to < 2 nm (figure 2A), towards the detection limit of DEER. Low temperature cw spectra (160 K) were acquired to complement the analysis. Spin normalized cw spectra clearly show dipolar broadening upon ATP binding visible as a decrease in the spectral intensity (figure 2A, inset in the third panel). The mean distance revealed by lineshape analysis (figure S2) is 1.6 nm (Gaussian dotted line in the P(r) panel of figure 2A), in line with the DEER data. ATP hydrolysis re-opens the periplasmic gate, restoring an apo-like conformation, as shown by DEER and cw EPR (figure S2).

Figure 3: Nucleotide-dependent movement of periplasmic (168-168) and cytoplasmic (141-141, 142-142) gates of BtuCD-F in LDAO micelles. Left panel) Normalized experimental data V(t)/V(0) and an exponentially decaying background arising from the distribution of remote spins (dashed lines), as fitted by DeerAnalysis2010. The DEER traces for the apo- and ATP- states of the periplasmic gate were detected at X band. All other traces were detected at Q band. The background was fitted with an exponential function (dimension 3 in DeerAnalysis2010) or stretched exponential function (dimension
3.3 Results

3.3.1 Opening and closing of the gates in detergent (LDAO)

All data presented above were obtained in proteoliposomes in the presence of the substrate binding protein BtuF in order to mimic as closely as possible the physiologically relevant state of the transporter. The question whether the membrane is necessary for the nucleotide-driven conformational switch was investigated by analyzing the complex solubilized in LDAO micelles. It is worth noting that the available crystal structures were also obtained from detergent-solubilized samples, which could impose conformational constraints during crystal formation. All detergent-solubilized samples were formed by addition of vitamin-bound BtuF in a two-fold excess to BtuCD. The room temperature cw spectra of the complex in LDAO showed an increased conformational flexibility in both gates with respect to the proteoliposomes, indicated by an increased mobility of the R1 side chains (figure S1). In detergent, the dynamics of the R1 side chain in BtuCD-F as detected by cw EPR are not significantly altered by the presence of nucleotides, probably due to the high intrinsic flexibility of the complex.
Figure 4: Nucleotide-dependent movement of periplasmic (168-168) and cytoplasmic (141-141 and 142-142) gates of BtuCD in LDAO micelles and liposomes. **Left panel** Normalized experimental data V(t)/V(0) and an exponentially decaying background arising from the distribution of remote spins (dashed lines), as fitted by DeerAnalysis2010. The DEER traces were detected at Q band (except for position 168 in liposomes). **Middle panel** Background corrected normalized form factor F(t)/F(0) and fit by the Tikhonov regularization with a regularization parameter α = 100 or 1000 (black dashed lines). **Right panel** Distance distribution P(r) obtained with DeerAnalysis2010. Color code as in figure 2 and 3.
3.3 Results

However, DEER analysis showed that the gates open and close similarly to the liposome-embedded samples, with the periplasmic gates closing, and the cytoplasmic gates opening upon ATP binding (figure 3). The restoration of the gates towards the apo-state following ATP hydrolysis was complete confirming that the detergent-solubilized transporter can undergo the nucleotide-induced conformational changes.

Unlike the proteoliposomes, the LDAO samples contained vitamin B\textsubscript{12} in a 1:1 molar ratio with respect to BtuF. In LDAO vitamin B\textsubscript{12} is released in the solution upon binding of BtuF to BtuCD. Nevertheless, to address a possible role of the vitamin to the observed gate movements, BtuCD-F complexes spin labeled at positions 168 were repetitively washed until they became colorless (no absorbance detected at 360 nm). DEER analysis of the samples revealed that the gate response is independent on the presence of vitamin B\textsubscript{12} (figure S6).

To verify that the observed movements of the TMDs are mediated through the nucleotide cycle catalyzed by NBDs, the non-hydrolyzable ATP analogue AMPPNP was added to the complex. AMPPNP locked the complex in an ATP-like state which was not further modified by incubation with MgCl\textsubscript{2} (figure S3). The EPR data obtained in liposomes and micelles suggest that ATP triggers an inward-facing conformation of BtuCD-F, opposite to what is validated for the type I importers by a large amount of biochemical and crystallographic data.

3.3.3 Transmembrane communication in the absence of substrate binding protein BtuF

Without BtuF, the characteristic open-close movement of the gates upon ATP binding and hydrolysis was not observed either in LDAO or in liposomes. The DEER data recorded in LDAO micelles reveal a very disordered periplasmic gate region, characterized by a broad distribution of distances between the R1 side chains (figure 4A). ATP binding induced a conformation with a slightly higher propensity towards shorter distances and ATP hydrolysis restored an apo-like conformation. The cytoplasmic gate (positions 141 and 142) shows analogous broad interspin distance distributions (figure 4C, E), which are only slightly affected by nucleotide binding. In proteoliposomes, the periplasmic gate of BtuCD shows as well a broad distance distribution which is shifted towards shorter distances by addition of ATP (figure 4B). The cytoplasmic gate (position 141) shows a main distance at around 3 nm which is only slightly affected by ATP binding (figure 4D). Overall, the data suggest that the gates are disordered in the absence of BtuF but some communication exists between NBDs and TMDs, in agreement with the futile ATP hydrolysis cycle detected in BtuCD. However, only in the presence of BtuF the gates adopt a well-defined conformation and fully accomplish the switch from the open to the closed state.
3.3.4 BtuF Nucleotide- and vitamin-dependent affinity to BtuCD

It has been shown that BtuCD or the preformed complex BtuCD-F cannot bind vitamin $B_{12}$ [58, 103]. The available structural and biochemical data suggest that BtuCD does not have a binding site for the substrate, unlike the type I importers. DEER and cw data in the presence and absence of BtuF clearly show that the substrate binding protein binds to the TMDs in all the three states of the transporter both in liposomes (no vitamin $B_{12}$) and in LDAO (vitamin $B_{12}$ to BtuF 1:1 ratio). Excess of ATP or ADP-MgCl$_2$ (up to 5 mM) had no effect on the affinity of BtuF for BtuCD at the micromolar protein concentrations used. Interestingly, a vitamin $B_{12}$ excess was shown recently to preferentially impair BtuF binding in the ATP-state of the transporter [58].

We conducted experiments using cw and pulse EPR to monitor the influence of a molar excess of vitamin $B_{12}$ to BtuF on the complex formation and the consequent effects on the periplasmic and cytoplasmic gates in detergent. We used as reporter moieties the spin labels at position 168 and 142. Position 141 was not investigated as the interspin distance distributions in the ATP-state are similar for BtuCD and BtuCD-F in detergent.

![Figure 5](image-url)

**Figure 5:** Effect of vitamin $B_{12}$ and ATP on the periplasmic gate in BtuCD-F in LDAO micelles. **A)** Inset shows room temperature cw EPR spectra of the periplasmic gate (168-168) in the presence of ATP without BtuF (grey), with 1:1 (black) and 1:50 (violet) molar ratio of BtuF to vitamin $B_{12}$. The characteristic immobile component (indicated by an asterisk) shows the interaction between BtuCD and BtuF. The arrow indicates the decrease of the immobile component at high vitamin concentrations. The horizontal scale represents 2 mT. Left panel, normalized form factors $F(t)/F(0)$ with the fit by Tikhonov regularization with a regularization parameter $\alpha = 100$ (dashed lines). Right panel, corresponding distance distribution $P(r)$ obtained with DeerAnalysis2010. **B)** Same experiments as in (A) performed in the cytoplasmic gate (142-142). The arrows indicate the reappearance of the distances in the ATP-state at high vitamin concentration. All DEER traces were recorded at Q band.

The room temperature cw EPR spectra of position 168 in BtuCD and BtuCD-F in LDAO (figure S1 and inset in figure 5A) show that binding of BtuF produces a characteristic peak
3.3 Results

In the low field region due to the reduced mobility of the R1 side chains (asterisk in the inset in figure 5A). By increasing the vitamin to BtuF molar ratio, this characteristic peak gradually vanishes in the presence of ATP. This suggests a gradual dissociation of BtuF from the periplasmic region of the transporter. DEER measurements showed that at high vitamin concentrations the periplasmic and cytoplasmic gates re-opens, adopting a more disordered conformation close to the one observed in the ATP-bound state of BtuCD (figure 5). In contrast, the same experiments performed in the ADP-state in both mutants showed a reduced dissociation of BtuF (figure S4). Attempts to conduct similar experiments in proteoliposomes were not conclusive due to the inside-out orientation of BtuCD-F and the difficulty to introduce excess of vitamin into the lumen of the liposomes.

3.4 Discussion

The present study reveals how the transmembrane gates in the vitamin $\text{B}_{12}$ transporter BtuCD-F respond to the presence of BtuF, substrate and nucleotides. The vitamin $\text{B}_{12}$ transporter is suggested to operate by an alternating-access mechanism in which the ATP-bound state shows an inward-facing conformation of the TM5 helices. The presence of BtuF is shown to be necessary for tight coupling between the NBDs and the transmembrane gates movements and the substrate is confirmed to modulate the complex dissociation.

BtuCD as shown by EPR adopts a dynamic conformation in the apo-state, with the gates overall displaying broader distance distributions than those expected by simulation of the interspin distances in the crystal structure. The comparison between simulations performed on the BtuCD crystal structure (PDB 1L7V) with MMM [105] and experimental distances detected in LDAO in the apo-state is presented in figure S5. The broad experimental distance distribution between positions 168 is in line with the simulation (figure S5). The experimental distances between positions 141 and 142 are longer than the simulated ones. To analyze the effects induced by the R1 neighboring residues on the simulated distances, an additional simulation was performed with the “any rotamers?” function available in MMM. All the 210 R1 rotamers of the MMM library were attached to the mutated cysteines, a condition which represents the absence of any steric constraints for the spin label rotamers in the structure but maintains the original position of the backbone to which the rotamers are attached. The latter simulation showed that the measured interspin distances in the cytoplasmic gate could not be completely reconciled with the available backbone conformation of the crystal. Among the possible explanations for the discrepancies are: i) the crystallization trapped the BtuCD transporter in the energetically favored conformation, ii) both gates are dynamic, which would explain the Hi1470/1 structure trapped in the opposite conformation, iii) the R1 side chain destabilizes the cytoplasmic gate in BtuCD. However, the last explanation appears unlikely because functionality tests showed that spin labeling did not affect ATPase activity (Table S1).
The simulations performed with MMM on the BtuCD-F structure (PDB 2QI9) (figure S5) showed a better agreement for both gates (positions 168 and 141) with the experimental data obtained in proteoliposomes in the apo-state (or ADP-state). For positions 142 simulated rotamers exist yielding a distance in the experimental range (black rotamers in figure S5C), however they are underrepresented in the simulation. We suggest that the semi-occluded asymmetric BtuCD-F crystal structure is a good representative for the complex in the apo- or ADP-state.

In this study we also confirmed by EPR that the substrate acts synergistically with ATP to decrease the affinity of BtuF for the transporter. Moreover, in presence of ATP and high vitamin to BtuF molar ratios, both periplasmic and cytoplasmic gates are shown to adopt a conformation close to the ATP-bound state of BtuCD. How to correlate the in vitro observations with the physiologically relevant conditions in vivo?

Uptake of the scarce molecule vitamin B₁₂ in the milieu by E. coli involves the high affinity outer membrane receptor BtuB [106], the cytoplasmic membrane bound TonB [107], the periplasmic substrate binding protein BtuF [51], and the transmembrane associated BtuCD [54]. The TonB-ExbB-ExbD complex is proposed to harness the energy of proton motive force to drive the release of vitamin B₁₂ from BtuB. BtuF, which seems to interact also with the TonB system [108], binds the released vitamin B₁₂ in the periplasm and forms a stable complex with BtuCD. ATP hydrolysis in BtuD is thought to provide energy for the release of vitamin B₁₂ through the translocation channel formed by BtuC with a mechanism which is still under investigation. BtuB is expressed at around 200-500 copies per cell [109] and it is post-transcriptionally regulated through alternate RNA structures induced by the binding of Ado-cobalamine to the 5’ untranslated (5’UTRs) region of the mRNA [110]. Unlike genes of other import systems, btuC₃D and btuF are transcribed independently. Both BtuCD [56] and BtuF [51, 52, 106] are expressed at very low levels and their expression is not regulated by vitamin B₁₂ [56, 111]. Although a quantitative analysis of the amount of BtuCD and BtuF in the cell has not yet been performed, about 3 vitamin B₁₂ binding sites per cell were reported in the periplasm of E. coli [106], which are potentially enough for the observed rate of vitamin B₁₂ import [112]. Considering the extreme stability of the BtuCD-F complex [58], essentially all of the BtuF in the cell will be in complex with BtuCD. Hence, the few copies of vitamin B₁₂ binding protein detected by White et. al. [106] may reflect the fraction of the free BtuF in the periplasm.

The scarce vitamin B₁₂ encountered by the bacteria in the milieu may be concentrated in the periplasm by the action of the high affinity BtuB and TonB system [53]. According to the in vitro observations, the synergistic action of intracellular ATP and increasing vitamin concentration in the periplasm can accelerate the dissociation of BtuF from the complex (figure 5 and Equation S1). It is worth mentioning that transmembrane potential or pH gradient in the cell could affect the vitamin to BtuF ratio required to dissociate the complex in vitro. Concomitantly to the BtuF release, ATP is hydrolyzed and the gates adopt the
more flexible conformation as observed in BtuCD (figure 4). The released BtuF can bind the available substrate preferentially reforming the complex with BtuCD in the apo- or ADP- state (figure 5 and Lewinson et al. [58]), to initiate a new productive cycle. We speculate that at this stage (absence of ATP) the vitamin is transiently released to the translocation pathway in the TMDs. Interestingly, radioactive traces of vitamin were found in membrane-embedded BtuCD-F only in the absence of ATP [103]. Though we did not follow the movement of substrate during the transport, our results strongly suggest that vitamin B_{12} is released to the cytoplasm upon ATP binding due to the opening of the cytoplasmic gate with concomitant closure of the periplasmic gate. The absolute requirement of ATP for vitamin B_{12} transport across the membrane, both in vivo and in vitro [8] further supports this model.

**Figure 6:** EPR distances and model of a productive vitamin transport cycle. **A)** Schematic description of the EPR-derived distances during the nucleotide cycle (apo-, ATP-, and ADP- states) for BtuCD (left panel) and BtuCD-F (right panel). The corresponding states of NBDs are indicated in black, red and green colors, respectively. The TM5 helices are highlighted in black. Spin-labeled positions are represented by yellow circles. Ovals were used for BtuCD to represent the large distance distributions detected. **B)** Model of a productive vitamin B_{12} transport in the cell. The vitamin B_{12}-bound BtuF interacts with the apo-state of BtuCD (1L7V), forming the BtuCD-F complex (model superimposed to 2Q19). Vitamin B_{12} is transiently released to the translocation channel. ATP binding to the NBDs inwardly opens the translocation channel, and the vitamin escapes into the cytoplasm. Simultaneously, the presence of excess vitamin in the periplasm helps to promote the dissociation of BtuF, restoring BtuCD to an outward facing conformation, ready to interact with another vitamin-bound BtuF.

Considering the mM concentration of ATP in the cytoplasm, BtuCD will be prevalently either in the ATP- or ADP- bound state. However, it is conceivable that the apo-state is also transiently populated during the exchange of ADP to ATP at the NBDs. A kinetic
analysis of the complex formation using surface plasmon resonance in the presence of 
Mg$^{2+}$-ATP suggested that on average BtuCD molecules reside longer in the ADP-bound state than in the ATP-bound or the transition-state-like (Mg$^{2+}$-ATP/vanadate) intermediates [58]. It has been an intriguing question for most ABC transporters, whether it is one or two molecules of ATP hydrolyzed by NBDs in a transport event. In liposomes BtuCD has a high basal ATPase activity which is further stimulated by addition of BtuF (Table S1). Considering this high basal ATPase rate and lack of any asymmetry at the NBDs upon BtuF binding [8], it is conceivable that both ATPs are hydrolyzed simultaneously. Based on the above considerations and the EPR data, which are schematically represented in figure 6A, we detail a model for a productive vitamin B$_{12}$ transport cycle mediated by BtuCD-F in the cell (Figure 6B).

The model proposed here points to a mechanistic difference in coupling the energy of ATP hydrolysis to substrate transport between type I and type II importers. The physiological implication of the model described here is that the BtuF affinity to BtuCD, thus the transport rate is tuned to the availability of vitamin in the periplasm: the higher the availability, the faster BtuF is released to the periplasm to bind the new substrate and deliver it to BtuCD preferentially in the apo- or ADP- states. In ButCD-F, the ATP-induced dimerization of the NBDs opens the translocation pathway towards the cytoplasm. A mechanistic implication of these observations is that the TMDs in ButCD-F and in the maltose transporter are driven in opposite directions along with the movements of NBDs. In both transporters the movement of TMDs along with NBDs is transmitted by the coupling helices. In BtuCD the NBDs are tilted with respect to the long axis of the BtuC dimer, positioning the coupling helices diametrically opposed [15].

Based on the BtuCD structure it was speculated that the closure of NBDs upon ATP binding would pull the coupling helices apart opening the TMDs towards the cytoplasm. The EPR data indeed suggest that binding of ATP opens the translocation pathway of BtuCD-F towards the cytoplasm. How the movement of the gates correlate to the overall TMD rearrangement and whether the mechanistic model proposed is valid for all members of the type II importers will be subject of further investigation.
3.5 Supporting Information

Figure S1: Dynamics of the cytoplasmic and periplasmic gates in BtuCD and BtuCD-F. **Left panel** Room temperature cw spectra of BtuCD solubilized in LDAO and spin labeled at the periplasmic (position 168, A) and cytoplasmic (141, B and 142, C) gates. Insets show the spectra of the protein reconstituted in liposomes. **Right panel** Spectra of BtuCD-F solubilized in LDAO. Insets show the spectra of the complex reconstituted in liposomes. Color codes as depicted in the figure: black, apo-state; red, incubation with ATP-EDTA; green, incubation with ATP and MgCl₂. The horizontal bars represent 2 mT. Asterisks indicate fractions of residual free label in solution.
Figure S2: Low temperature cw EPR analysis. Low temperature spin normalized cw EPR spectra of BtuCD-F spin labeled at position 168 (A), 141 (C) and 142 (E) in liposomes or detergent as indicated. The fit performed with DIPFIT [81] (dotted lines) is presented in panels (B), (D), (F) for the spectra detected in liposomes or detergent as indicated. The Gaussian distance distributions are presented in the bottom panels. For positions 141 and 168, the fits of the LDAO-solubilized samples are not shown because they were not very informative, due to the broad distance distribution centered at around 2 nm (limit of cw sensitivity). The linewidth parameters used for the fitting of position 168 and 141 were obtained from the corresponding low temperature spectra of BtuCD in the apo-state, characterized by distances > 2 nm as detected by DEER (figure 4). For position 142, only the LDAO-solubilized samples were analyzed because in liposomes the DEER analysis was sufficient to extract all distance information. The linewidth parameters for position 142 were extracted from the fit of the low temperature spectrum of BtuCD-F in the ATP-state showing a narrow 2.4 nm distance distribution in the DEER analysis (figure 3).
### Figure S3: Effect of AMPPNP on the cytoplasmic gate in BtuCD-F. DEER analysis on BtuCD-F spin labeled at position 141 solubilized in LDAO in the presence of 1 mM AMPPNP (magenta) and after addition of 5mM MgCl$_2$ (green). **Left**) Normalized experimental data $V(t)/V(0)$ and exponentially decaying background arising from a 3D distribution of remote spins (black dotted lines), as fitted by DeerAnalysis2010 [87]. **Middle**) Background corrected normalized form factors $F(t)/F(0)$ and fit by the Tikhonov regularization with a regularization parameter $\alpha = 100$. **Right**) Distance distribution $P(r)$ obtained with DeerAnalysis2010. The opening of the cytoplasmic gate induced by AMPPNP is not reverted to the closed apo-like-state by incubation with MgCl$_2$.

### Figure S4: Effect of vitamin B$_{12}$ and ADP on the periplasmic gate in BtuCD-F in LDAO micelles. **A**) Inset shows room temperature cw EPR spectra of the periplasmic gate (168-
in the presence of ADP without BtuF (grey), with 1:1 (black) and 1:50 (violet) molar ratio of BtuF to vitamin B$_{12}$. The characteristic immobile component in BtuCD-F (indicated by an asterisk) shows the interaction between BtuCD and BtuF. In contrast to the ATP-state, the immobile component does not decrease at high vitamin concentrations. Left panel, normalized form factors F(t)/F(0) with the fit by the Tikhonov regularization with a regularization parameter $\alpha = 100$ (dashed lines). Right panel, corresponding distance distribution P(r) obtained with DeerAnalysis2010. B) Same experiments as in (A) performed for the cytoplasmic gate (142-142). All DEER traces were recorded at Q band. In the periplasmic gate a 50 fold excess of vitamin to BtuF in presence of ADP-MgCl$_2$ does not induce any observable dissociation of the complex. In the cytoplasmic gate under similar conditions a partial dissociation of the complex is observed, but less than that induced by ATP (figure 5). The observed difference in the extent of complex dissociation under similar conditions might be due to the selective effect of the mutations and spin labeling of the corresponding positions. The overall observations in both gates are consistent with a higher dissociation of BtuF from the complex in the presence of ATP and vitamin excess.

Figure S5: Comparison between experimental DEER data and interspin distances simulated with MMM (http://www.epr.ethz.ch/software/index). A) R1 rotamers simulated on the BtuCD structure (PDB code 1L7V) presented as transparent grey stick representation. The positions of the N-O midpoints are indicated by magenta balls. B) Simulated distance distributions (black dotted lines) for the investigated positions superimposed to the experimental distance distributions in the apo-state (black straight lines). The measured distances show deviations from the X-ray structure. To reveal the influence of neighboring amino acids on the predicted distance distributions, an additional
simulation was performed with the “any rotamers?” function available in MMM (grey dotted line). This simulation uses all the 210 R1 rotamers of the library to produce a distance distribution as close as possible to the experiment. For the periplasmic gate of BtuCD the latter simulation predicts a distance distribution in better agreement with the experiment. However, for the cytoplasmic gate the discrepancy still persists. C) Simulated distance distributions (black dotted lines) for the investigated positions in BtuCD-F (PDB code 2QI9) superimposed to the experimental distance distributions in the apo- (black straight lines) and ATP- (red lines) states. The simulated distances for the gates in BtuCD-F show the best agreement to the experimental ones measured in the apo states. The inset at the bottom shows the underrepresented simulated rotamers (black) attached at positions 142 which are responsible for distances in the 1.6-2.0 nm range. The remaining rotamers (yellow) seem not to be populated in proteoliposomes. Moreover, in MMM the rotamer simulation for each site is performed independently, thus close rotamers (< 1 nm distance) which have steric clashes are not removed prior to the calculation of the distance distribution, which is an additional reason for the overrepresented short distances.

Figure S6: Movement of the periplasmic gate (168-168) of BtuCD-F in LDAO in the absence of vitamin. Left panel) Normalized experimental data V(t)/V(0) and an exponentially decaying background arising from the distribution of remote spins (dashed lines), as fitted by DeerAnalysis2010. All the DEER traces were detected at Q band. The background was fitted with an exponential function (dimension 3 in DeerAnalysis2010). Middle panel) Background corrected normalized form factor F(t)/F(0) and fit by the Tikhonov regularization with a regularization parameter $\alpha = 100$ (black dashed lines). Right panel) Distance distribution P(r) obtained with DeerAnalysis2010. Color codes: black, apo; red, ATP; sea green, ADP and MgCl$_2$. The observed distance distributions are essentially the same as in presence of vitamin B$_{12}$ (figure 2A). Minor changes might be due to the removal of some BtuF molecules during the washing steps performed to remove vitamin B$_{12}$. 
Figure S7: Post-hydrolytic states induced by ATP-MgCl₂ or ADP-MgCl₂ in the periplasmic gate (168-168) of BtuCD-F in LDAO. Comparison between the distances in the apo-state and in the post-hydrolytic states of BtuCD-F obtained after incubation with ATP and MgCl₂ (green) or addition of ADP and MgCl₂ (sea green). The results show that both methods induced a protein conformation very similar to the apo-state (black). The sample incubated with ATP and MgCl₂ shows some residual short distances attributed to the ATP-state (arrow). (Left panel) Modulation-depth-normalized form factors $F(t)/F(0)$ and fit by the Tikhonov regularization with a regularization parameter $\alpha = 100$ (black dashed lines). (Right panel) Distance distribution. For clarity, $P(r)$ intensities were normalized at 2.5 nm (vertical dotted line). Color codes: green, ATP-MgCl₂; sea green, ADP and MgCl₂; black, apo-state.

3.6 Supporting table

Table S1. ATPase activity of the spin-labeled mutants

<table>
<thead>
<tr>
<th></th>
<th>Mutant</th>
<th>ATPase activity BtuCD (nmol Pi/min/mg protein)</th>
<th>ATPase activity BtuCD-F (nmol Pi/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDAO</td>
<td>141R1</td>
<td>710</td>
<td>1060</td>
</tr>
<tr>
<td></td>
<td>142R1</td>
<td>420</td>
<td>660</td>
</tr>
<tr>
<td></td>
<td>168R1</td>
<td>1230</td>
<td>1350</td>
</tr>
<tr>
<td>LPS</td>
<td>141R1</td>
<td>750</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td>142R1</td>
<td>440</td>
<td>490</td>
</tr>
<tr>
<td></td>
<td>168R1</td>
<td>450</td>
<td>670</td>
</tr>
</tbody>
</table>
Spin labeling at positions 141 and 168 did not change the activity in LDAO as compared to the wild-type transporter [103]. For position 142, a 50% reduction in ATPase activity is observed. The ATPase activity in liposomes are slightly higher than those reported before for the wild type transporter probably due to the fact that the reconstitution efficiency and the protein concentration are determined here based on the spin concentration and the labeling efficiency and not by the intensity of the bands in SDS gels. The protein concentration obtained by EPR was confirmed by UV absorbance at 280 nm using the DDM-solubilized proteoliposomes. Errors due to reproducibility of the ATPase activity were calculated to be in 10-20% range.

3.7 Supporting text

1. $\text{BtuCD-F } \overset{K_d \approx 10^{-10}}{\leftrightarrow} \text{BtuCD + BtuF}$

2. $\text{F-vitB}_{12} \overset{K_d \approx 10^{-6}}{\leftrightarrow} \text{F + vitB}_{12}$

3. $\text{BtuCD-F + vitB}_{12} \overset{K_d \approx 10^{-2}}{\leftrightarrow} \text{BtuCD + F-vitB}_{12}$

4. $\frac{[\text{BtuCD}]}{[\text{BtuCD-F}]} = 10^{-2} \frac{[\text{vitB}_{12}]}{[\text{F-vitB}_{12}]}$

**Equation S1:** Equilibrium constants for BtuCD-F association in presence of ATP. Effect of Vitamin $B_{12}$ and ATP on the association of BtuCD and BtuF in presence of ATP. The dissociation constants are taken from a previous study [58]. A 100 fold excess of vitamin to BtuF in the presence of ATP produces a 1:1 ratio of BtuCD:BtuCD-F, thus both fractions are observable by EPR. The stability of the BtuCD-F complex is increased by three orders of magnitudes in the absence of ATP ($K_d \approx 10^{-13}$ M for reaction 1).
This work is submitted for publication (Joseph et. al. 2013) with Vladimir M Korkhov, Maxim Yulikov, Gunnar Jeschke and Enrica Bordignon as the co-authors. V.M.K prepared the BtuCD E82C mutant. All the other biochemical and EPR works were done by me.

4. Conformational transitions of the vitamin $\text{B}_{12}$ ABC importer in liposomes in the course of substrate translocation

4.1 Introduction

ABC transporters are one of the largest families of membrane proteins, mediating the transport of diverse substances across the membrane. They are involved in cellular processes like nutrient uptake, drug resistance, immune response, osmotic homeostasis, and lipid and cholesterol trafficking [1, 57]. They consist of two transmembrane domains (TMDs) which determine the substrate specificity, connected to two nucleotide binding domains (NBDs) which bind and hydrolyze ATP. Bacteria possess both ABC importers and exporters, while only ABC exporters are found in eukaryotes. Based on TMD architecture, ABC importers are classified into type I, type II [45], and type III [113].

A complete elucidation of the ‘alternating-access’ model of action involved in the transport cycle of type I ABC importers was obtained by combining crystal structures of the maltose importer from $\text{E. coli}$ in different states in the presence and absence of substrate binding protein [28, 35-37, 95] with other biochemical and biophysical data [60]. Here we investigate the nucleotide and substrate-dependent conformational changes of BtuCD, a type II importer mediating uptake of vitamin $\text{B}_{12}$ with the help of the binding protein BtuF. The first two nucleotide-free crystal structures (BtuCD and BtuCD-F) suggested that the translocation mechanism might be different from the ‘alternating-access’ model widely accepted for type I ABC importers [8, 15]. Importantly, the BtuCD-F apo crystal structure showed an occluded translocation channel with no space for vitamin $\text{B}_{12}$. The ATP-induced opening of the cytoplasmic gate I (position 141 in TM5 helices) suggested by changes in the spin label’s mobility with continuous wave EPR [114] and proven by double electron-electron resonance (DEER) [39] hinted at an inward-facing translocation channel, which is opposite to what was observed for type I ABC importers [35].
Recently the AMPPNP (a non-hydrolysable ATP analogue)-bound BtuCD-F was crystallized using the ATP-ase deficient E159Q mutant stabilized by cysteine cross linking of the NBDs (N162C). This structure confirmed the swing-out motion of the cytoplasmic gate I in TM5 helices, but found it coupled to the closure of the short loops (called cytoplasmic gate II) between TM2 and TM3 [40]. These loops sealed the translocation pathway, creating a cavity in the center of the channel, which could harbor a vitamin B$_{12}$ molecule. In line with that, radioligand trapping experiments showed enhanced amounts of vitamin B$_{12}$ in liposome-reconstituted transporters only in presence of AMPPNP [40]. Based on these results and on the fact that ATP hydrolysis was already proven to be required for vitamin import by BtuCD-F in proteoliposomes [40, 103], it was proposed that the release of the substrate occurs after ATP hydrolysis through a not yet identified inward-facing conformation [40], resembling the inward-facing structure of HI1470/1 from Haemophilus influenzae [16]. A previous surface plasmon resonance study showed that the presence of vitamin B$_{12}$ and ATP strongly accelerates the complex dissociation rate, thus a different mechanism in which vitamin B$_{12}$ is directly translocated in the apo-state and ATP binding is required only for BtuF dissociation was proposed [58].

Intriguingly, on one side ATP increases BtuF dissociation when vitamin is present, but on the other side it creates a cavity in the translocation channel to trap vitamin. The aim of this work is to use EPR spectroscopy to bridge the “mechanistic” gap between the observed enhanced complex dissociation in the presence of vitamin and ATP and the trapping of substrate in BtuCD-F under the same conditions during a translocation cycle in liposomes.

It is known that protein-mediated transmembrane transport can be tuned by the presence of the membrane[59]. DEER is a powerful tool to reveal functional intermediate states in ABC transporters [39, 63, 98-100, 102], symporters [115] or ion channels [116, 117] and it can be applied in a native-like lipid environment providing inter-residue distance information in the 1.5-6 nm range [70]. Here we used site-directed spin labeling and DEER complemented with biochemical data to study concomitantly the nucleotide- and substrate-dependent dynamics of BtuCD-F complex formation and the structural changes in the translocation channel in liposomes.

The BtuCD-F translocation channel consists of residues from TM5-TM5a helices, the loop between TM2 and TM3 (cytoplasmic gate II) and a few residues from TM3 and TM8 helices; the channel is further flanked with TM10 residues (figure 1A-B). We attached nitroxide-based spin labels (MTSL, (1-Oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl) methyl methanethiosulfonate) to several key positions along the channel and one additional Gd(III)-DTPA-based spin label (maleimido-monoamide DTPA (diethylene triamine pentaacetic acid) [77]) in BtuF (figure 1A).

Our results show that a BtuCD-F complex can be formed in presence of AMPPNP in liposomes with a sealed translocation channel, as seen in the crystal, which probably harbors the vitamin B$_{12}$. Combining the EPR data on intermediates in BtuCD and BtuCD-F
with the available crystal structures helps to elucidate important details of the mechanism of substrate translocation.

4.2 Materials and Methods

4.2.1 Protein Expression and Purification

BtuF was purified as described before [103]. The concentration of vitamin-loaded BtuF was determined by absorption at 361 nm. Cysteine mutations on BtuF were performed on the wild-type gene and for BtuCD a “cys-less” construct [8] was used. BtuCD was overexpressed in E. coli BL-21-(DE3) Gold cells. The frozen cells were solubilized in 1% LDAO (lauryldimethylamine-N-oxide, Anatrace) in 50 mM Tris-HCl (pH 7.5) containing 25 mM imidazole-HCl (pH 8.0), 5 mM β-mercaptoethanol and 500 mM NaCl. The cells were sonicated using a Sonics Vibra-Cell sonicator, centrifuged for 30 minutes at 40,000 x g. The supernatant was loaded onto a Ni-NTA column, pre-washed with 50 mM Tris-HCl (pH 7.5) containing 0.1% LDAO, 25 mM imidazole-HCl (pH 8.0), 5 mM β-mercaptoethanol and 500 mM NaCl. The column was washed with 50 mM Tris-HCl (pH 7.5) containing 0.1% LDAO and 500 mM NaCl, followed by 90 mM imidazole-HCl (pH 8.0) in the same buffer. Protein was eluted with 200 mM imidazole in 50 mM Tris-HCl (pH 7.5) containing 0.1% LDAO and 500 mM NaCl and was immediately desalted with HiPrep desalting column (GE Healthcare) into 50 mM Tris-HCl (pH 7.5) containing 0.1% LDAO and 500 mM NaCl.

4.2.2 Spin labeling and ATP-ase assay

For Gd(III) labeling, BtuF was incubated overnight at 4°C with three fold molar excess of maleimido DTPA loaded with Gd(III) ions (100% loading efficiency). BtuCD was concentrated to 15-20 µM and a 20-40 fold molar excess of MTSL ((1-oxyl-2,2,5,5, -tetramethyl-d-3-pyrroline-3-methyl) methanethiosulfonate) was added at room temperature while shaking. Free MTSL was removed using PD10 desalting columns (GE Healthcare) and the protein was concentrated with centricons (50 kDa cutoff). Spin labeling efficiency was calculated by double integral analysis of the CW EPR spectra using 4-Hydroxy-TEMPO as standard. Spin-labeling efficiencies were calculated to be in the 70-100% range for the mutants prepared. To form detergent-solubilized BtuCD-F, BtuF was added to the spin-labeled BtuCD at a molar ratio 2:1. The ATP-ase assay on the spin-labeled mutants was performed as described before [103].

4.2.3 Liposome Reconstitution of BtuCD and BtuCD-F

Before reconstitution, BtuCD and BtuCD-F were concentrated to 20-30 µM and reconstitution was performed directly from LDAO, using an established protocol which
gave an inside-out orientation (NBDs facing outside) for more than 90% of the BtuCD molecules.\cite{39, 103} Following reconstitution, protein concentration in PLS was calculated using the known spin-labeling efficiency of the mutant and the measured spin-concentration. A 1:1000 molar ratio of BtuCD to lipids was used. The liposomes used contained \textit{E. coli} polar lipid extract and Egg L-a-phosphatidylcholine at a 3:1 ratio (w/w).

4.2.4 SDS-PAGE for observation of complex formation

Reconstituted BtuCD (8 μM) was extruded (10 times) through 400 nm membrane and 5 times frozen and thawed with or without AMPPNP-Mg\textsuperscript{2+} (1 mM AMPPNP and 1 mM MgCl\textsubscript{2}). As a control, empty liposomes were extruded as well in the presence of Mg\textsuperscript{2+}. Vitamin-loaded BtuF (8 μM) was added with an additional 5 times freezing and thawing. Following incubation, the liposomes were pelleted and the supernatant was checked for BtuF with SDS-PAGE.

4.2.5 Vitamin B\textsubscript{12} trapping in proteoliposomes

Liposomes (at 10-20 mg/ml) containing BtuCD (10-20 μM) were 10 times extruded through a 400 nm membrane, AMPPNP or ADP-Mg\textsuperscript{2+} (1 mM AMPPNP or ADP, 250 μM MgCl\textsubscript{2}) was added and 5 times frozen and thawed. These liposomes were pelleted and resuspended to a final concentration of 150-200 μM BtuCD. BtuF or BtuF-vitamin B\textsubscript{12} were added at the indicated concentrations and incubated for 1 hour at 25°C. Here we observed only the inside-out oriented BtuCD molecules as there was no extrusion or freezing and thawing step after addition of BtuF. Following incubation, liposomes were pelleted and the supernatant was checked for vitamin B\textsubscript{12} by detecting the absorption at 550 nm (extinction coefficient of 8739 M\textsuperscript{-1} cm\textsuperscript{-1}).

4.2.6 Sample preparation for EPR measurements

BtuCD and BtuCD-F in LDAO or reconstituted in liposomes were prepared in the apo-state (absence of nucleotides). The ATP-bound intermediate was prepared incubating BtuCD (50-100 μM) at 4°C for 10 minutes with 1 mM ATP and 50 μM EDTA. The ADP-state was obtained incubating the sample at 4°C for 10 minutes with 1 mM ADP and 2 mM MgCl\textsubscript{2}. We were able to achieve a nearly complete binding of the nucleotides, due to a uni-directional inside-out orientation of BtuCD and BtuCD-F in liposomes following reconstitution \cite{39}.

For Gd(III)-NO DEER, reconstituted BtuCD (300-500 μL of 15 or 10 μM BtuCD for the 82 and 168 mutants, respectively, 10-20 mg/ml lipid) nitroxide labeled at position 82 or 168 in BtuC was extruded with 400 nm membrane, 5 times frozen and thawed with 15 μM vitamin-loaded BtuF-Gd(III) labeled at position 138 with or without 1 mM AMPPNP. The liposomes were pelleted and resuspended in about 25 μL of buffer. For DEER
measurements, 10% v/v deuterated glycerol was added to the samples before snap freezing them in liquid nitrogen. DEER measurements were performed as described below.

To trap a transition state analog, reconstituted BtuCD (15 μM BtuCD, 10-20 mg/mL lipid) was extruded with 400 nm membrane, 5 times frozen and thawed with vitamin-loaded BtuF (1:1 to BtuCD), 10 mM ATP, 10 mM vanadate and 10 mM MgCl₂ and incubated at 37°C for 1 hour. In other experiments where complex formation was studied in liposomes in presence of vitamin or nucleotides, reconstituted BtuCD (8-10 μM, 300-500 μL) was was extruded (10 times) through 400 nm membrane and 5 times frozen and thawed with nucleotides, followed by BtuF (with or without vitamin B₁₂) with an additional 5 times freezing and thawing at the indicated concentrations. Following incubation, the liposomes were pelletted and resuspended in about 25 μL buffer. For DEER, 10% v/v deuterated glycerol was added to the samples before snap freezing them in liquid nitrogen.

4.2.7 EPR Measurements

Low-temperature EPR spectra for interspin distance determination were recorded in EPR quartz capillaries (3 mm outer diameter, sample volume 30 μL) on a CW EPR Bruker spectrometer Elexsys E500 at 160 K. The spectra were detected with 100 kHz field modulation, 0.08 mW microwave power, 0.25 mT modulation amplitude. Dipolar broadened EPR powder spectra were fitted with the software DIPFIT.[81] The linewidth parameters of the reference non-dipolar-broadened spectrum in LDAO or in liposomes were obtained by fitting the spectrum in a nucleotide state characterized by distances >2 nm as judged from DEER, as indicated in the corresponding figure legends. In the case such condition was not met for one specific mutant, another spin-labeled position in the same environment (LDAO or liposomes) was used instead. The dipolar-broadened spectra were fitted by fixing all parameters, except the maximum hyperfine coupling Azz, the interspin distance and the width of the distance distribution (Gaussian model). DEER measurements were performed on a home-made Q-band spectrometer (34-35 GHz) equipped with a TWT amplifier (150 W) and a home-made rectangular resonator enabling the insertion of sample tubes with 3 mm outer diameter [104]. Dipolar time evolution data were acquired using the four-pulse DEER experiment. All DEER measurements were performed at 50 K. All pulses were set to 12 ns and deuterium nuclear modulations were averaged by increasing the first interpulse delay by 16 ns for 8 steps [86]. For NO-NO DEER, the ELDOR frequency was set at the maximum of the echo-detected field swept spectrum, 100 MHz higher than the observed frequency. For Gd(III)-NO DEER, the pump pulse was set at the maximum of the echo-detected field swept spectrum for NO, 300 MHz higher than the observer frequency for Gd(III) ions. The background of the normalized DEER primary data (V(t)/V(0)) was fitted and the resulting normalized secondary data (F(t)/F(0)) were converted by a model-free Tikhonov regularization to distance distributions with the software DeerAnalysis2011 [87]. The simulation of the possible spin label rotamers attached at a position was performed using the Matlab program package MMM (http://www.epr.ethz.ch/software/index) based on a rotamer library approach [105].
4.3 Results

4.3.1 Conformation of the translocation channel in BtuCD-F in the absence of nucleotides

Spin labels were attached at key positions in the translocation channel in BtuC (figure 1A): the periplasmic end of TM5 (position 165), the periplasmic (position 307) and cytoplasmic (position 322) ends of TM10 and the cytoplasmic gate II (positions 82 and 85). To characterize the conformation of the channel in the apo-state, we used Q-band DEER complemented with low-temperature continuous wave EPR on BtuCD-F in liposomes (figure 1C, black lines). The DEER data already published [39] on the periplasmic gate between TM5 and TM5a (positions 168) and the cytoplasmic gate I in TM5 (position 141) are presented in the general context of the translocation channel movements.

All spin-labeled proteins were found to be active based on the ATP-ase assays (table S1). The BtuCD-F complexes analyzed in figure 1 were pre-formed in LDAO detergent (1:2 BtuCD to BtuF, $K_d = 10^{-13}$ M [58]) and subsequently reconstituted, which leads to a liposome preparation without vitamin B$_{12}$ [8, 39], with the majority of the transporters having the inside-out orientation (NBDs facing outside) [39].

The primary DEER data and the fits corresponding to figure 1 (except for position 168 [39]) are shown in the supplementary material (figures S1-6). Comparison between the experimental distance distributions (figure 1C, black) with those simulated with the software MMM [105] on the BtuCD-F apo-crystal structure (figure 1C, black dotted) shows good agreement for positions 82, 141, 168, 322. The deviations between the experimental data and the simulations are within the accuracy achievable with the adopted rotamer library approach (3-3.5 Å) [118].

For positions 165 (distance extracted by continuous wave EPR) and 307 we observed some discrepancies between simulated and experimental data, which may be due to the tight location of the labels (figures S3E, S4I) for which only one or a few rotamers were significantly populated. In the latter cases the predicted rotamer position is very sensitive to the resolution of the underlying structure and to the approximation used in the simulation approach; moreover labeling those sites may induce protein distortion [105]. Interestingly, for these two sites in LDAO-solubilized BtuCD-F complexes, the agreement between simulations and experiments was found to be very good (figures S3E, S4I), showing that the membrane environment influences the distance distributions at these labeled sites. Overall, the analysis suggests that in liposomes BtuCD-F adopts a conformation consistent with that observed in the crystal, with an occluded translocation channel.
Notably, the interspin distances measured in the BtuCD-F apo-state in LDAO micelles vs liposomes showed a longer mean distance (2.6 nm in LDAO vs 1.3 nm in liposomes) between the periplasmic TM5 ends (position 165), a broader and longer distance between the cytoplasmic ends of TM10 (position 322, 4.5 nm in LDAO vs 3.5 nm in liposomes), and a decreased 82-82 distance in the cytoplasmic gate II (1.7 nm in LDAO vs 2.1 nm in liposomes), highlighting the influence of the environment on the observed interspin distances (figure S7A).

Figure 1: Interspin distances in BtuCD-F apo- and AMPPNP- states in liposomes. a) X-ray structure of BtuCD-F apo-state with the translocation channel highlighted (PDB 2QI9). The TM5 helices are in green, the cytoplasmic gate II (loops between helices TM2 and TM3) in gold and the TM10 helices in red. The Cα atoms of the spin-labeled positions are highlighted. b) X-ray structure of the translocation channel in AMPPNP-BtuCD-F (PDB 4FI3). c) Experimental distance distributions obtained from DEER or by convolution of the low-temperature CW spectra (position 165) are shown in black (apo-BtuCD-F) and in red (ATP-EDTA-BtuCD-F). Vertical dashed lines guide the eye for comparison. The MMM simulations on the corresponding crystal structures are shown in dotted lines (black dotted, PDB 2QI9; red dotted, PDB 4FI3). Asterisks on positions 82 and 307 in presence of ATP indicate distances corresponding to a fraction of BtuCD molecules (see figure 2).
The data for positions 141 and 168 were published before [39]. The primary data are shown in figures S1, S3-5.

4.3.2 ATP binding at NBDs induces major conformational changes in the translocation channel

Addition of ATP-EDTA to BtuCD-F reconstituted in liposomes (in the absence of vitamin) resulted in significant conformational changes in the translocation channel as reported by the key spin-labeled residues (figures 1C, red lines). Comparable results were obtained with AMPPNP as shown for several positions (figures S6). The periplasmic gate on the short loop between TM5 and TM5a (position 168) closed, whereas the distances between positions 165 at the TM5 helical end remained unchanged (consistent with the small C-C distance increase of 0.6 Å observed in the corresponding BtuCD-F crystals [8, 40]). The distance between the periplasmic ends of TM10 (positions 307) is increased by about 1 nm upon ATP binding as visible from the DEER analysis (figure 1C) as well as from the low temperature CW data (see figure S4H). The cytoplasmic ends of TM10 helices (positions 322) show only a slight rearrangement of the bimodal distance distribution towards longer distances (figure 1C, visible also from the primary traces in figure S5D).

At the cytoplasmic end of TM5 (cytoplasmic gate I, position 141) the mean distance increased from 2.0 to 2.5 nm [39], consistent with the swing-out motion of the TM5 observed in the crystal [40], and concomitantly the cytoplasmic gate II (position 82) closed, as detected by a change in mean distance from 2.1 to 1.6 nm. Spin labels attached at position 85 in the TM2-TM3 loop, which is one of the most conserved residues in this gate for the type II ABC importers of the FecD family [40], also showed a characteristic distance decrease upon ATP binding (figure S2), confirming the results obtained with the reporter label at position 82. Comparison between experimental distances and MMM simulations on the AMPPNP-BtuCD-F structure (figure 1C, red dotted lines) reveal a good agreement except for the periplasmic positions 165 and 307. The discrepancies in these cases may be due to the same reasons detailed above for the apo-BtuCD-F (figures S2F, S3J), and to the fact that the electron density of the side chains in these regions is not well defined [40].

The tight coupling observed in liposomes (figure 1C) between ATP binding and closure of the cytoplasmic gate II (position 82) was not detected when the BtuCD-F complexes were analyzed in LDAO detergent micelles. In fact, the short distance between positions 82 characteristic of the sealed cytoplasmic gate II in the AMPPNP-BtuCD-F was already present in the apo-BtuCD-F in detergent and was not further modified by binding of ATP (figure S1). However, the labels attached at positions 85 in the gate II showed a change from a narrow distance centered at 2.45 nm in the apo-BtuCD-F to a broad distribution from 5 nm down to 1.3 nm (figure S2). This indicates that the loop between TM2-TM3 has a different response to ATP binding in LDAO than in liposomes.
Focusing on the distance distribution obtained for cytoplasmic gate II, we found that the distance at 1.6 nm detected in the AMPPNP-BtuCD-F in liposomes is in good agreement with the simulations on the AMPPNP-BtuCD-F crystal; however this peak was accompanied by an additional reproducible broad distance distribution at 3 nm (asterisk in figure 1C, position 82). A closer examination shows that the 3 nm distribution nicely corresponds to that obtained in the ATP-bound BtuCD (figure 2A, figure S1), suggesting that ATP or AMPPNP induces partial dissociation of BtuCD-F complexes in liposomes, in agreement with the SDS-PAGE data of Lewinson et al [58]. The distance associated with the presence of BtuCD is completely different from that observed in apo-BtuCD-F (figure 1C), ruling out the possibility that it arises from a fraction of BtuCD-F, which did not interact with the nucleotides. A similar effect, though with lower peak resolution (due to distance overlapping) was observed for position 307 as well (figure 2B). The partial complex dissociation observed with the reporter position 82 in liposomes at BtuCD-F concentrations in the 50-100 micromolar range used in the experiments presented in figure 1C indicates a dissociation constant in the same micromolar range, which is significantly different from the value of 6.2·10^{-11} M detected in LDAO-solubilized samples [58]. For all other positions investigated, the two fractions (corresponding to BtuCD and BtuCD-F) appearing due to partial complex dissociation cannot be distinguished by DEER due to significant overlap of the two distance distributions.

**Figure 2:** ATP-induced dissociation of BtuCD-F complex in liposomes. Distance distributions in ATP-BtuCD-F (red) for positions 82 (a) and 307 (b) (distributions taken from figure 1). The distance distribution of the corresponding BtuCD preparation in the presence of ATP-EDTA is presented in dotted blue lines (taken from figure 3).

### 4.3.3 ATP-hydrolysis restores the apo-like conformation of the translocation channel

The post-hydrolytic ADP-Mg^{2+}-bound BtuCD-F is an intermediate in the vitamin B_{12} transport cycle, for which no structural information yet exists. Our results in liposomes reveal that the periplasmic gate (position 168 [39]) and the cytoplasmic gate II (position 82, figure S1) reopen after ATP hydrolysis and the cytoplasmic gate I (position 141, figure S1) goes towards the apo-state-like closed conformation. Positions 307 and 322 (TM10, figures S4, 5) as well have distance distributions similar to the BtuCD-F apo-state. Altogether this suggests that following ATP hydrolysis, the translocation channel adopts a conformation similar to that trapped in the BtuCD-F apo-crystal (figure S8), thus it probably has no space to accommodate vitamin B_{12}. 


4.3 Results

4.3.4 Response of BtuCD to nucleotide cycle

The apo-BtuCD was crystallized with an outward-facing translocation channel [93]. BtuCD alone has basal ATP-ase activity in both liposomes and LDAO micelles [103] but it is still unclear how the nucleotide cycle at NBDs is coupled to conformational changes in TMDs in the absence of BtuF. To address this question, we obtained distance constraints between key spin-labeled sites on apo-, ATP- and ADP-Mg$^{2+}$-bound BtuCD in liposomes (figure 3 and figures S1-5). Comparison between the experimental apo-distances (figure 3, black lines) and those simulated with MMM (figure 3, black dotted lines) on the BtuCD structure reveals a good agreement, except for the cytoplasmic gate I (position 141 [39]), suggesting that the conformation in liposomes is consistent with the available structure.

Figure 3: Distance distributions in liposomes for BtuCD in apo- (black), with ATP-EDTA (red) or with ADP-Mg$^{2+}$ (green). The distance distributions obtained from DEER or by convolution of the low-temperature CW spectra (positions 165 and 307) are shown (primary data in figures S1, S3-5). The simulated distances on the apo-BtuCD crystal structure (PDB 1L7V) are presented in dotted lines. The data for positions 141 and 168 were published [39].

There exists some communication between NBDs and the translocation channel also in the absence of BtuF (figure 3), however in general the mean interspin distances and the changes induced by nucleotides are different than those observed in BtuCD-F (figures S1-5). Upon ATP binding to BtuCD (figure 3, red lines) the interspin distances between positions 141 and 168 were already shown to be slightly affected [39] and some changes are visible as well for position 165 and 307 (figure 3 and figures S3, 4) in liposomes. Interestingly, the characteristic decrease in distance detected in BtuCD-F between the spin labels at positions 82 in the cytoplasmic gate II is not visible in BtuCD reconstituted in liposomes (figure 3). However, we could detect a distance decrease between positions 85 in BtuCD liposomes (figure S2), similar to that observed in BtuCD-F, suggesting a different conformation of this loop in BtuCD compared to BtuCD-F. In the presence of ADP-Mg$^{2+}$ the distances are found to be similar to those detected in the apo-state (figure 3, green lines).
In LDAO distance changes are also visible upon ATP binding for most positions investigated in BtuCD, however the distance distributions especially for positions 82, 85, 165 and 322 differ from those detected in liposomes (figures S1-5).

### 4.3.5 Trapping of vitamin B₁₂ following BtuF-BtuCD association

The AMPPNP-BtuCD-F crystal was obtained with the ATP-ase deficient E159Q mutant, which was locked into the AMPPNP-bound conformation with cysteine cross-linking at the NBDs (N162C). The sealed cavity along the translocation channel revealed from this structure does not have vitamin inside. Vitamin trapping experiments demonstrated specific trapping of vitamin in presence of AMPPNP supplementing the crystallographic data [40]. However neither the conformation of the channel nor the existence of a BtuCD-F complex could be monitored in these experiments. The presented DEER data prove that in liposomes the AMPPNP-bound BtuCD-F complex exists with a closed cytoplasmic gate II (figure 1), as observed in the crystal structure, but it remains unknown whether presence of vitamin B₁₂ may modify this conformation.

### 4.3.6 Vitamin and nucleotide effects on BtuCD-F complex formation and conformation of translocation channel

It has been shown that vitamin and ATP enhance complex dissociation both in detergent-solubilized and reconstituted transporters [58]. However, vitamin could be trapped only in the presence of AMPPNP, as shown here and previously [40]. Here we studied in detail the effects of vitamin and nucleotides on the conformation of the translocation channel and on the interaction between BtuF and BtuCD in liposomes. We focus on the conformation of the cytoplasmic gate II (position 82), which is responsible for the sealing of the channel, thus the trapping of the vitamin. This gate shows characteristic and distinguishable distance distributions in the different intermediate states (3 nm in BtuCD; 2.1 nm in apo-BtuCD-F and 1.6 nm in ATP-BtuCD-F, figures 1C and 3, see position 82). Thus it is possible to monitor complex dissociation and sealed or open conformation of the translocation channel from the DEER distance distribution for this position.

For this type of experiments, we prepared samples by adding one equivalent of BtuF (± vitamin B₁₂) to BtuCD (spin-labeled at position 82) (10 µM) reconstituted in liposomes followed by 5 times freeze-thaw cycles to achieve a homogenous distribution of additives and a 50:50 orientation of BtuCD molecules [119]. After sample centrifugation, DEER was measured on the pellet resuspended with a minimal volume of buffer. The DEER traces in the apo- as well as in the ADP-BtuCD-F (figure 4C and figure S9) slightly differ in the absence and presence of vitamin B₁₂. The obtained distance distributions show a slight decrease of the 2.1 nm peak intensity (associated with 82-82 distance in BtuCD-F) accompanied by an increase of the 3 nm peak intensity (associated with 82-82 distance in
4.3 Results

BtuCD), suggesting a slightly reduced complex formation under the experimental conditions used. In the presence of AMPPNP and vitamin B$_{12}$ a more pronounced decrease of the peak intensity at 1.6 nm (associated with the AMPPNP-BtuCD-F fraction) and a concomitant increase of the 3 nm peak intensity (AMPPNP-BtuCD fraction) was observed. This suggests that despite enhanced dissociation, the sealed conformation of the translocation channel is maintained in a fraction of BtuCD-F complex even in presence of vitamin B$_{12}$. An SDS-PAGE performed with the supernatants of the DEER samples (figure 4B) further confirmed the DEER results: in the absence of nucleotides, the band corresponding to the BtuF molecular weight was almost below detection limit, while a significant amount of BtuF was present in the supernatant in the presence of AMPPNP-Mg$^{2+}$, indicative of decreased complex formation.

**Figure 4:** Complex dissociation and vitamin trapping. **a)** 13 µM vitamin-loaded BtuF was incubated with liposome-reconstituted BtuCD in apo-, with ADP-Mg$^{2+}$- or with AMPPNP. The supernatant was checked for vitamin B$_{12}$ by detecting the absorption at 550 nm. **b)** 8 µM vitamin B$_{12}$-loaded BtuF were added to BtuCD reconstituted in liposomes (8 µM) in the absence or presence of AMPPNP-Mg$^{2+}$. The supernatant was checked for BtuF via SDS-PAGE. In the absence of AMPPNP, BtuF was below detection limit (first lane). In the presence of AMPPNP, a significant amount of the BtuF was detected (second lane, BtuF added before AMPPNP-Mg$^{2+}$, third lane, reversed order), similar to that in the control sample (fourth lane). **c)** Left panels, modulation depth-normalized form factors $F(t)/F(0)$ (solid lines) and fit (grey dotted lines) for position 82 in the apo- and AMPPNP- states after incubation with unloaded BtuF (black) and vitamin-loaded BtuF (magenta). Right panels, obtained distance distributions. The black and green asterisks indicate the distance distribution corresponding to BtuCD and BtuCD-F, respectively. **d)** Gd(III)-NO DEER on BtuF138Gd(III)-BtuC168R1 confirms enhanced complex dissociation. The inset shows schematic view on the position of Gd(III) and NO spin labels on BtuCD-F.
To confirm that the small fraction of the BtuCD-F-like distance distribution between the gate II labeled sites observed in the presence of AMPPNP and vitamin (figure 4C, distance centered at 1.6 nm in the AMPPNP-Mg$^{2+}$ panel) represents the fraction of transporters with bound BtuF, it is necessary to detect the binding of BtuF to BtuCD under the same experimental conditions. To this end, we labeled BtuF (S138C) with maleimido-DTPA-Gd(III) and the periplasmic gate BtuCD (position 168) with MTSL and performed Gd(III)-NO DEER [74] at Q band to follow BtuF-BtuCD interaction. This experiment would not be very informative if for all positions the nitroxide-based MTSL were used because of overcrowding of distance distributions in triply-labeled BtuCD-F. The Gd(III)-NO experiment has already been tested on model systems [75, 78] and soluble proteins [75, 76] and it can be performed selectively without contaminations from NO-NO distance distributions. A distance distribution centered at 4 nm (Cβ-Cβ distance of 2.6 and 2.5 nm in the apo- and AMPPNP-crystals) was detected between BtuF and position 168 in BtuCD via Gd(III)-NO DEER (figure 4D) using Gd(III) as observer spin and the nitroxide as pump spin [74]. The modulation depth ($\lambda$) of the Gd(III)-NO DEER trace gives direct information on the presence and extent of complex formation: the smaller the $\lambda$, the smaller the fraction of BtuCD-F complexes in the sample. Despite the significantly reduced modulation depth in presence of AMPPNP and vitamin (figure 4D), in agreement with NO-NO DEER data on position 82, we could confirm that the BtuCD-F complex indeed exists in liposomes.

Further, incubating the Gd(III)-labeled BtuF with BtuCD carrying the nitroxide labels at the cytoplasmic gate II (position 82), we could selectively detect on the same sample in the presence of vitamin and AMPPNP the Gd(III)-NO distance > 6 nm corresponding to the AMPPNP-BtuCD-F complex (Cβ-Cβ distance of 6.7 nm in the AMPPNP-crystal) as well as the NO-NO distance at 1.6 nm characteristic of the sealed cytoplasmic gate II in AMPPNP-BtuCD-F (figure 4 and figure S10A,B). Combining the EPR results with our vitamin trapping experiments, it is highly likely that this small fraction of BtuCD-F complex harbors the vitamin B$_{12}$ inside the translocation channel.

**Figure 5:** Simultaneous detection of BtuCD-F complex and sealed cytoplasmic gate II. a) Gd(III)-NO DEER between BtuF138Gd(III) and BtuC82R1 confirms the existence of BtuCD-F complex. b) NO-NO DEER between BtuC82R1 and BtuC82R1 on the same sample confirms the existence of the sealed cytoplasmic gate II. The inset shows a schematic view on the position of Gd(III) and NO spin labels on BtuCD-F.
4.4 Discussion

We additionally performed an additional experiment using vitamin-loaded wild type BtuF and liposome-reconstituted BtuCD carrying the nitroxide labels at the cytoplasmic gate II (position 82), trapped in the catalytic transition-state intermediate by vanadate-induced nucleotide trapping [58]. The vanadate-trapped BtuCD-F mimics the intermediate immediately after ATP hydrolysis, with the phosphate group still present. Interestingly, the 82-82 distances measured in this transition-state intermediate were almost indistinguishable from those detected in presence of AMPPNP (figure S10C).

4.4 Discussion

The sets of EPR data validate key features of the three available crystal structures of the vitamin B$_{12}$ ABC importer as representatives of the structures in liposomes and provide additional information on ATP- and ADP-Mg$^{2+}$-BtuCD, and on BtuCD-F in the ADP-Mg$^{2+}$-bound form and in the transition state intermediate.

Interaction between unloaded-BtuF with the apo- or ADP-BtuCD leads to the formation of a stable complex at the micromolar concentration used. In this condition, the majority of the experimental interspin distances are found to be consistent with the occluded translocation channel in the BtuCD-F apo-crystal structure. In particular, cytoplasmic gate I (position 141) is closed, while gate II (positions 82, 85) is open (figure 1, figures S1-2). In this conformation, the crystal structure exhibits no space for a vitamin molecule in the channel [8]. The ADP-Mg$^{2+}$-bound BtuCD-F in liposomes was found to be very similar to the apo-conformation (figures S1, S3-5 and S8). Interestingly, the presence of vitamin (1:1 to BtuF) for the apo- or ADP-BtuCD-F only slightly affected complex association as judged by DEER (figure 4). Despite the complex is readily formed in liposomes, interaction of loaded BtuF with apo- or ADP-BtuCD triggers a non-productive cis-release of the substrate into the supernatant.

Upon ATP binding to BtuCD-F in the absence of vitamin, we could observe that cytoplasmic gate I opens (position 141) [39], while cytoplasmic gate II closes (82-82 distance of 1.6 nm, figure. 1C; 85-85 distance of 1.5 nm, figure S2), the latter being responsible for sealing of the translocation channel, as observed in the AMPPNP crystal structure. In particular, when analyzing the response of gate II (position 82), we found that upon ATP binding to BtuCD-F at micromolar concentrations a distance distribution characteristic of the same labeled site in BtuCD appeared (figure 2), indicative of a dissociation constant in micromolar range in the absence of vitamin in membrane environment. In contrast, in LDAO unloaded BtuF was found to form an extremely stable complex with BtuCD in presence of ATP ($K_d$ 6.2·10$^{-11}$ M) [58].

Interaction of vitamin-loaded BtuF with the AMPPNP-bound BtuCD leads to measurable amount of trapped vitamin B$_{12}$ in the liposome fraction, in agreement with previous
radioligand trapping experiments [40] and to an enhanced complex dissociation, detected by SDS-PAGE and by DEER the reporter spin label at position 82 in the cytoplasmic gate II, in line with previous findings [58]. Here, using an orthogonal labeling strategy in the presence of vitamin B$_{12}$, we could correlate in the same liposome sample the existence of BtuCD-F complexes (via BtuF-BtuCD distance determination) to the presence of a sealed cytoplasmic gate II characteristic of AMPPNP-BtuCD-F (via 82-82 distance determination). Though vitamin-loaded BtuF in presence of AMPPNP had the lowest affinity to BtuCD, we never observed by EPR a complete dissociation of the complex at the micromolar concentrations used, which suggests a BtuF-BtuCD dissociation constant in the micromolar range.

We thus conclude that a productive translocation event starts with interaction of vitamin-loaded BtuF with ATP-bound BtuCD (model in figure 6). Korkhov et al. [40] proposed that once the AMPPNP-BtuCD-F complex harbouring the vitamin is formed, ATP hydrolysis proceeds and once the hydrolysis products are released an inward-facing conformation will be generated to squeeze out the substrate. In presence of vitamin we found the same distance distributions in the cytoplasmic gate II in the AMPPNP-bound form and in the transition state intermediate, which narrows down the time window for the appearance of this putative inward-facing conformation: the substrate is suggested to be released into the cytoplasm simultaneously with the phosphate release, but before the Mg$^{2+}$-ADP- or apo-BtuCD-F is formed (model in figure 6). In fact the latter intermediates can be considered post- or pre-translocation intermediates, respectively, because they are characterized by an occluded translocation channel with the cytoplasmic gate I (position 141) closed, as discussed above. We suggest that the differential timings between the reopening of the cytoplasmic gate II and the closure of gate I may be the key mechanistic steps for vitamin to be expelled from the channel. Coupling between these two motions may be also critical for avoiding that small molecules from the cytoplasm enter the corresponding large cavity. After ADP is exchanged with ATP (present in mM concentrations in the cell) the complex dissociation is enhanced, which would allow the next cycle to start with the capture of another vitamin B$_{12}$ by the BtuF released in the periplasm and its subsequent rebinding to the ATP-BtuCD (figure 6).

**Figure 6:** Schematic description of the translocation mechanism for vitamin B$_{12}$. The TM5 helices are shown in green, the cytoplasmic gate II in gold. A productive transport cycle begins with interaction of vitamin B$_{12}$-loaded BtuF with ATP-bound BtuCD. Vitamin B$_{12}$ is released during phosphate release, before the Mg$^{2+}$-ADP-BtuCD-F is achieved. Subsequent release of ADP and rebinding of ATP induces complex dissociation to start another import cycle.
4.4 Discussion

All experiments used here to draw conclusions on the translocation mechanism were performed in liposomes, which mimic the native membrane environment. EPR studies on MalFGK₂-E and the lipid flippase MsbA showed that they have a similar conformation in liposomes and detergents [61, 100, 102], whereas EPR studies on the aspartate transporter Glt₆ₙ revealed that a membrane environment favors conformations different from those observed in detergent micelles [120, 121]. Thus, structural differences between detergent and liposome preparations depend on the protein and the type of detergent. This study on BtuCD-F shows that some positions in the channel are characterized by different distance distributions and extent of distance changes in the two environments, highlighting the importance of such comparative analysis for reliable interpretation of the EPR data.

In this work we highlight characteristic features in the mechanism of action of the type II importer BtuCD-F in liposomes which coexist with common properties observed in other ABC importers, such as the ATP-induced closure of NBDs and coupling helices and the requirement of ATP-hydrolysis for substrate release. Further work is needed to test whether the latter properties are universal for the ABC transporters family.
4.5 Supporting Information

82-82

**Figure S1:** Q-band DEER data for positions 82 (a–d) and 141 (e) on BtuCD or BtuCD-F in LDAO and liposomes. **Left** normalized experimental data V(t)/V(0) and background fits (dotted lines) by DeerAnalysis2011 [87]. **Middle** background corrected normalized form...
factors $F(t)/F(0)$ (solid lines) and fits by Tikhonov regularization (dotted lines). Right) obtained distance distributions. The asterisk in panel (d) highlights the distance centered at about 3 nm characteristic of a fraction of ATP-BtuCD. The inset in panel (b) shows the validation of the distance distributions in the ATP-bound BtuCD in liposomes performed with DeerAnalysis2011. The major peak centered at about 3 nm is accurately described by the analysis, while the peaks at longer distances are characterized by a larger degree of uncertainty, as is the case for other peaks appearing at distances > 4 nm in the traces detected in BtuCD and BtuCD-F in liposomes. For position 141, higher quality Q-band DEER data in liposomes are presented, for which the X-band data was published before [39]. Color code: apo (black), ATP-EDTA (red) and ADP-Mg$^{2+}$ (green).
Figure S2: Q-band DEER traces for position 85 on BtuCD (a, b) or BtuCD-F (c, d) in LDAO or liposomes as indicated. Panel description as in figure S1. In panels (c) and (d) only the apo- and ATP-BtuCD-F are shown. In panel (b) and (d) the simulations performed on the available structures are superimposed in dotted lines. The simulated distances < 1.5 nm in the 4FI3 structure reflect the close vicinity of the two 85 side chains. A 1 nm threshold limit is applied in the presented distance distribution. e) Left panel, region of the translocation channel in BtuCD (PDB 1L7V, TM5-TM5a helices, green; TM10 helices, red; loop TM2-3, yellow; view from the cytoplasm). The R1 (MTSL) rotamers calculated for
positions 85 with the software MMM[105] are presented in ball and stick representation. The yellow spheres represent the position of the nitroxide mid-point and the radius of the sphere corresponds to the population of the rotamers. f, g) Analogous region of the translocation channel in BtuCD-F (PDB 2QI9) and AMPPNP-BtuCD-F (PDB 4FI3). The simulated rotamers in each site are computed separately. In the AMPPNP-BtuCD-F (PDB 4FI3) the simulated rotamers largely collide, which decreases the accuracy of the simulations. The two engineered cysteines at positions 85 were shown to cross link both in the apo- and in the AMPPNP-BtuCD-F in LDAO [40] and the C -C distance is only 5.4 Å. Thus the information obtained with the 85-85 pair needs to be treated carefully.
Chapter 4. Conformational transitions of the vitamin B\textsubscript{12} ABC importer

Figure S3: Q-band DEER traces and low temperature CW spectra for position 165 on BtuCD (A, B) or BtuCD-F (C, D) in LDAO and liposomes. a, c) Panel description as in figure S1. b, d) Left, spin-normalized low temperature CW EPR spectra of the samples in liposomes. The corresponding non dipolar broadened spectra obtained for the same mutant in the LDAO-solubilized apo-BtuCD or BtuCD-F are shown to highlight the dipolar broadening visible in the liposome samples. Middle, fit of the simulated spectra performed with DIPFIT\cite{DIPFIT} (dotted lines) to the experimental ones (colored lines). Right, obtained Gaussian distance distributions. The line width parameters used for the fitting were
obtained from the fit of the low temperature spectrum of apo-BtuCD in liposomes labeled at position 141[39], which showed distances > 2 nm. Color code: apo (black), ATP-EDTA (red) and ADP-Mg\(^{2+}\) (green). **e) Left panel,** region of the translocation channel in BtuCD-F (PDB 2QI9: TM5-TM5a helices, green; TM10 helices, red; loop TM2-3, yellow; view from the periplasm with BtuF removed for clarity). The R1 (MTSL) rotamers calculated for positions 165 with the software MMM[105] are presented in ball and stick representation. The green spheres represent the position of the nitroxide mid-point and the radius of the sphere corresponds to the population of the rotamers. **Right panels,** comparison between simulated (dotted grey lines) and experimental (black lines) distance distributions for positions 165 in BtuCD-F detergent-solubilized (LDAO) and reconstituted in liposomes (lipo) in the apo-state. The clearly bimodal distribution of rotamers in one BtuC subunit (black circles highlight the two fractions) and the presence of only one rotamer populated in the other BtuC subunit make the simulated distance distribution extremely narrow and strongly bimodal and can be the reasons for the discrepancies observed between experimental and simulated distances. **F) Panel description as in (E).** **Right panel,** region of the translocation channel in AMPPNP-BtuCD-F (PDB 4FI3). **Left panel,** comparison between the simulated and experimental distance distributions in the AMPPNP-bound BtuCD-F in liposomes and LDAO. Similarly to what was observed in the apo BtuCD-F structure, both sites 165 show a restricted space available for the rotamers, thus the simulated distance distribution is very narrow. This is a possible source for the discrepancies between experimental and simulated distributions.
Figure S4: Q-band DEER traces and low temperature spectra for position 307 on BtuCD (A-D) or BtuCD-F (E-H) in LDAO and liposomes. Panel description as in figure. S1. For the DIPFIT analysis, the line width parameters of the low temperature spectrum of position 141 in BtuCD apo-state in LDAO or liposomes [39] were used for the fitting of LDAO or...
liposome data, respectively. Color code: apo (black), ATP-EDTA (red) and ADP-Mg^{2+} (green). I) Left panel, region of the translocation channel in BtuCD-F (PDB 2QI9: TM5-TM5a helices, green; TM10 helices, red; loop TM2-3, yellow; view from the periplasm with BtuF removed for clarity). The R1 (MTSL) rotamers calculated for positions 307 with the software MMM [105] are presented in ball and stick representation. The red spheres represent the position of the nitroxide mid-point and the radius of the sphere corresponds to the population of the rotamers. Right panels, comparison between simulated (dotted grey lines) and experimental (black lines) distance distributions for positions 307 in BtuCD-F detergent-solubilized (LDAO) and reconstituted in liposomes (lipo) in the apo-state. The clearly bimodal distribution of rotamers in one BtuC subunit (black circles highlight the two fractions) and the presence of only one rotamer populated in the other BtuC subunit make the simulated distance distribution extremely narrow and strongly bimodal and can be the reasons for the discrepancies observed between experimental and simulated distances. J) Panel description as in (I), the asterisk denotes a fraction of the distance associated with BtuCD (see figure 2). Right panel, region of the translocation channel in AMPPNP-BtuCD-F (PDB 4FI3). Left panel, comparison between the simulated and experimental distance distributions in the AMPPNP-BtuCD-F in liposomes and LDAO. Similarly to what was observed in the apo BtuCD-F structure, both sites 307 show a bimodal distribution of the few rotamers populated, thus multiple peaks appear in the simulated distance distribution. The tight nature of the labeled sites is a possible source for the discrepancies between experimental and simulated distributions.
**Figure S5:** Q-band DEER for position 322 in LDAO and liposomes. **Left** normalized experimental data V(t)/V(0) and background fits (dotted lines) by DeerAnalysis2011 [87]. **Middle** background corrected normalized form factors F(t)/F(0) (solid lines) and fits by Tikhonov regularization (dotted lines). **Right** obtained distance distributions. Color code: apo (black), ATP-EDTA (red) and ADP-Mg^{2+} (green).
Figure S6: Comparison of ATP-EDTA and AMPPNP- states for positions 82, 141, 307 and 322 in BtuCD-F in liposomes (A-D). Samples were prepared by adding Mg$^{2+}$-AMPPNP to BtuCD-F (50-100 µM) reconstituted in liposomes. **Left**, normalized experimental data $V(t)/V(0)$ for the Mg$^{2+}$-AMPPNP-BtuCD-F. The background fits by DeerAnalysis2011 are presented as dotted lines. **Middle**, background corrected normalized form factors $F(t)/F(0)$ (solid lines) and fits by Tikhonov regularization (dotted lines). **Right**, obtained distance distributions (black lines) superimposed to the corresponding data for ATP-BtuCD-F (dotted, taken from figure S1, 4-5).
Figure S7: Comparison of distance distributions between LDAO and liposomes for BtuCD and BtuCD-F in the apo-form or in presence of ATP-EDTA. Distance distributions obtained by Tikhonov regularization of the background-corrected DEER traces or by convolution of the low-temperature CW spectra in BtuCD and BtuCD-F in liposomes (lipo) and LDAO (primary data and fits are presented in figures S1-S4) in the apo- (A) and ATP-state (B). The major detergent-induced distance changes are highlighted by arrows.
Figure S8: Distance distributions in BtuCD-F in the presence of ADP-Mg\(^{2+}\) compared to available BtuCD-F crystal structures. Distance distributions obtained by Tikhonov regularization of the background-corrected DEER traces (green lines) in BtuCD-F in liposomes in the presence of ADP-Mg\(^{2+}\) (primary data and fits are presented in figures S1, S3-5). The simulated distances obtained on BtuCD-F apo- (dotted black lines) and AMPPNP- (dotted red lines) crystal structures are shown for comparison.
Figure S9: Effect of vitamin B$_{12}$ and/or nucleotides on the distance distributions in BtuCD-F in liposomes. Reconstituted BtuCD (8 μM final), MTSL-labeled at position 82 was extruded (10 times) through a 400 nm membrane, 5 times frozen and thawed in the absence of nucleotides or with ADP-Mg$^{2+}$ or AMPPNP-Mg$^{2+}$ (1 mM AMPPNP or ADP and 1 mM MgCl$_2$), followed by addition of BtuF or vitamin-loaded BtuF (8 μM final) with an additional 5 times freezing and thawing. Proteoliposomes were pelleted and resuspended in about 25 μL buffer for DEER measurements. Left), normalized experimental data obtained at 50 K using BtuCD spin labeled at position 82 in the apo- (A), with ADP-Mg$^{2+}$- (B) or with AMPPNP-Mg$^{2+}$ (C). Panel description as in figure S1. Color code: with vitamin B$_{12}$ (magenta), without vitamin B$_{12}$ (black). The green and black asterisks indicate the characteristic distance distribution corresponding to BtuCD-F and BtuCD, respectively. D) Enhanced dissociation of the BtuCD-F complex in presence of vitamin and AMPNP was further verified with orthogonal spin labeling and Gd(III)-NO DEER at 10 K. A Gd(III) tag on BtuF (position 138) and a nitroxide (NO) label at position 168 on BtuC were introduced.
Chapter 4. Conformational transitions of the vitamin B$_{12}$ ABC importer

BtuCD-F complex dissociation in presence of AMPPNP and vitamin B$_{12}$ is revealed from the reduced modulation depth of the form factors (central panel). Color code: with vitamin B$_{12}$ and AMPPNP (cyan), with vitamin B$_{12}$ only (black).

Figure S10: Demonstration of the existence of BtuCD-F complexes with closed cytoplasmic gate II (position 82) in liposomes in presence of vitamin B$_{12}$ and AMPPNP or ADP-vanadate. Using an orthogonal spin-labeling strategy with a Gd(III) tag on BtuF (position 138) and a nitro oxide (NO) label at cytoplasmic gate II (position 82), BtuF-BtuCD (complex formation) and BtuC-BtuC (gate conformation) distances were detected on the same sample by Q-band DEER at 10 and 50 K, respectively. BtuCD (15 μM final, 500 μL) MTSL-labeled at position 82 (cytoplasmic gate II) was extruded (10 times) through 400 nm membrane, 5 times frozen and thawed with AMPPNP (1mM), followed by addition of vitamin-loaded BtuF (15 μM final) with an additional 5 times freezing and thawing. Proteoliposomes were pelleted and resuspended in about 25 μL buffer for DEER measurement. A) Gd(III)-NO DEER (cyan) at 10 K and B) NO-NO DEER (black) at 50 K. The green and black asterisks indicate the characteristic distance distribution corresponding to BtuCD-F and BtuCD, respectively. C) The experiment was performed using wild type BtuF and BtuCD reconstituted in liposomes in the presence of vitamin, ATP, and vanadate to trap the transition state intermediate. The distance distribution obtained (green) was found to be very similar to that obtained in the presence of AMPPNP under the same conditions (black, as in panel B).
4.6 Supporting table

<table>
<thead>
<tr>
<th>Spin-labeled position</th>
<th>ATP-ase activity (nmol min⁻¹ mg⁻¹)</th>
<th>Spin labeling efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>82 (TM2-3 loop)</td>
<td>970</td>
<td>1110</td>
</tr>
<tr>
<td>85 (TM2-3 loop)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>165 (TM5)</td>
<td>1190</td>
<td>1200</td>
</tr>
<tr>
<td>307 (TM10)</td>
<td>1350</td>
<td>1600</td>
</tr>
<tr>
<td>322 (TM10)</td>
<td>1640</td>
<td>2080</td>
</tr>
</tbody>
</table>

Table S1: ATP-ase activity assay for the investigated spin-labeled mutants in LDAO. The assay used in this study was performed as described before using three replicates for each measurement [39]. Errors due to reproducibility of the ATPase activity were calculated to be in the 10-20% range. All the spin-labeled mutants have ATP-ase activities comparable to the wild type [103] and the other mutants (positions 141 and 168) described before [39]. The error in the spin labeling efficiency determination by double integration of the room temperature cw-EPR spectra and protein concentration is in the 10% range. n.d: not determined
5. Summary and Outlook

Despite the common architecture revealed by the available crystal structures, there are increasing evidence that a unifying transport mechanism, in which ATP binding at the NBDs induces an outward-facing TMD conformation and after hydrolysis the TMDs revert to an inward-facing conformation, does not apply to all transport processes mediated by ABC transporters. The EPR data obtained with liposome-reconstituted BtuCD, a type II ABC importer, confirm that the nucleotide cycle at the NBDs induces distinct conformational changes in the TMDs, which are different from those reported for type I ABC importers and exporters. The DEER data presented here strongly suggest that the inward-facing core translocation channel with a sealed cavity (in which vitamin B$_{12}$ can be trapped), as observed in the AMPPNP-BtuCD-F crystal structure represents the conformation present in a native-like membrane environment. Combining the DEER data for several of the possible intermediates in the translocation cycle (for which no crystal structures are available) with biochemical experiments, it is proposed that a productive transport cycle begins with interaction of vitamin B$_{12}$-loaded BtuF with ATP-BtuCD. This interaction leads to the trapping of vitamin B$_{12}$ as shown here and by the group of K. Locher [40]. The data indicate that the trapped vitamin B$_{12}$ must be released after ATP hydrolysis, but before Mg$^{2+}$-ADP-BtuCD-F is formed.

The results presented in this thesis show that the mechanism for vitamin transport previously proposed by Lewinson et al. [58], in which a vitamin molecule is transported in the absence of ATP (figure A1), cannot be valid in liposomes. The EPR data prove that in apo- or ADP-BtuCD-F there is no space for vitamin B$_{12}$ inside the transporter and that interaction of vitamin-loaded BtuF with apo- or Mg$^{2+}$-BtuCD results in a non-productive cis release of the substrate. The authors reported that the complex dissociation is enhanced in the presence of ATP ($K_d$ changes from $10^{-13}$ to $10^{-11}$ M) or vitamin B$_{12}$ ($K_d$ increases from $10^{-13}$ to $10^{-8}$ M) and no complex formation could be detected when both ATP and vitamin B$_{12}$ were present ($K_d$ higher than $10^{-4}$ M). These conclusions were drawn from results obtained with LDAO detergent-solubilized transporters. The DEER experiments with liposome-reconstituted BtuCD confirm that the affinity for complex formation is the lowest when both vitamin and ATP are present. However, a more than 7 orders of magnitude higher dissociation of BtuCD-F going from ATP to ATP+vitamin B$_{12}$ as reported by Lewinson et al. was not observed in liposomes as we were able to detect BtuCD and BtuCD-F under both conditions (ATP and ATP+vitaminB$_{12}$) at micromolar concentration.
The sequence of events for vitamin transport suggested by this EPR study on liposome-reconstituted transporters is in line with the model (figure A1 B, C) proposed by Korkhov et al. [40]. The EPR data help to better define the different phases in the transport cycle such as the initiation of a productive transport cycle, which requires the interaction of vitamin-loaded BtuF with ATP-BtuCD, the release of vitamin B$_{12}$, which is suggested to occur between the transition state intermediate and the Mg$^{2+}$-ADP-BtuCD-F and the dissociation of the BtuCD-F complex, which is favored by the replacement of ADP with ATP at the NBDs.

During this thesis a comparative analysis of the DEER data for BtuCD and BtuCD-F in liposomes and LDAO detergent micelles was also performed. Some positions in the translocation channel are found to be characterized by different distance distributions and extent of distance changes in the two environments. In particular, the cytoplasmic gate II, which closes or opens the translocation pathway in the ATP- or ADP- (and apo-) bound forms respectively, was found to be persistently locked into the closed conformation in LDAO micelles. Also, the presented results strongly suggest that the affinity of interaction between BtuF and BtuCD (and the extent of the influence of vitamin and nucleotides), which is very important for the understanding of the transport mechanism, might be different in liposomes as compared to that in LDAO detergent micelles. Though such discrepancies as described here can be detergent and protein specific, the results obtained here show that with membrane proteins whenever possible it is advisable to perform a comparative analysis of structural and functional properties between detergent micelles and liposomes.

The available data and the results presented here strongly suggest that vitamin B$_{12}$ might be trapped in the cavity in ATP-BtuCD-F. In order to locate vitamin B$_{12}$ in this cavity, a spin labeled-vitamin B$_{12}$ was synthesized during this thesis work, carrying a TMPO nitroxide radical. Preliminary data show that the TEMPO-vitamin B$_{12}$ binds both to BtuF, although with lower affinity than the wild type substrate (figure A2) and to the outer membrane vitamin B$_{12}$ transporter BtuB (figure A3). In the future we plan to perform DEER experiments using TEMPO-vitamin B$_{12}$ in combination with spin-labeled BtuC to locate the substrate inside the BtuCD-F complex at different stages of the transport cycle. Based on the results obtained for BtuCD-F, it will be of general interest to further investigate whether other type II ABC importers such as the heme transporter HmuV and the putative metal chelate importer Hi1470/71 as well function with a similar mechanism.

This thesis demonstrates a successful application of continuous wave and pulsed EPR techniques to probe conformational changes in a membrane protein complex. X-ray crystallography stands alone as a technique for atomistic resolution of structures of membrane protein complexes. To fully describe the mechanism of action of a membrane protein, one of the challenges in crystallography is to obtain structures of the different states populated during the protein functioning, for example during substrate translocation in the case of ABC transporters. However, the requirement to extract the proteins into
detergent micelles and stringent crystallization conditions may not allow formation of suitable crystals for all possible protein conformations or may cause artifacts in membrane protein crystal structures. Förster resonance energy transfer (FRET) is another potential technique to study conformational changes through distance measurement in biomacromolecules. FRET has the great advantage that it can be performed at ambient temperatures and at its best on a single molecule. However, depending on the distance to be measured, FRET requires distinct pairs of bulky chromophores which may influence the protein’s structure or stability. Though NMR is advancing towards solving atomistic resolution structures for larger protein complexes, it is still far from solving structures of membrane protein complexes of the size of the ABC importer BtuCD-F. In EPR, DEER experiments provide only sparse interspin distances between spin-labeled sites, in analogy to FRET. In contrast to FRET, measurements are performed at cryogenic temperatures, which may lead to selection of lower energy intermediates of the conformational landscape. When crystal structures are available, this problem could be partially alleviated by comparing the experimental distances with those simulated using rotamer library approaches. Compared to the other above mentioned methods DEER also has several advantages. Precise distance information (mean distance and width of the distribution) can be obtained on biomacromolecules irrespective of their size. Thus, distance measurements can be performed on protein complexes reconstituted in membranes or in principle even inside the whole cell. The EPR labels are smaller than the FRET chromophores and the same type of labels can be used at several sites independently of the distance to be measured, as long as it is in the sensitive range. In most cases the EPR probes do not perturb the protein’s structure or function. Distance can be detected in different states of the protein, giving an overall picture of the conformational changes; for example in an ABC transporter in the process of substrate translocation as demonstrated in this thesis. With the recent technical advancements, distances from 1.5 up to 10 nm have been precisely measured on soluble proteins and it is very likely that the current upper limit of 6 nm will be extended to 10 nm in membrane proteins as well in the near future. Clearly, EPR has established itself as a powerful tool in structural biology and, combined with other techniques, it can provide important information on structural and functional properties of membrane proteins during their functioning.
A Appendix

A. Lewinson et. al. 2010 [58]

B. Korkhov et.al. 2012[40]

C. Joseph et. al. 2013 [submitted]

Figure A1: Proposed vitamin B₁₂ translocation mechanisms. A) The vitamin B₁₂ molecule is transported in the absence of ATP and ATP binding is required for BtuF dissociation only. The EPR data obtained with BtuCD-F suggests that this mechanism is not valid in liposomes as the translocation channel in apo- and Mg²⁺-ADP-BtuCD-F has an occluded conformation with no space for vitamin as also observed in the apo-BtuCD-F crystal structure [8]. In agreement with this observation, vitamin B₁₂ was released into the supernatant upon interaction of vitamin B₁₂-loaded BtuF with Mg²⁺-ADP-BtuCD reconstituted into liposomes B) Vitamin B₁₂ is trapped in the translocation channel in ATP-BtuCD-F and it is released following ATP hydrolysis in a putative inward-facing
conformation. **C** This model is in agreement with the mechanism proposed in B. A productive translocation cycle can begin only with interaction of a vitamin B$_{12}$-loaded BtuF with ATP-BtuCD. Vitamin is trapped in ATP-BtuCD-F as in model B. The trapped vitamin can be released after ATP hydrolysis (during phosphate release), but before the Mg$^{2+}$-ADP-BtuCD-F is formed as it is characterized by an occluded translocation channel. Replacement of ADP with ATP induces BtuCD-F complex dissociation to start another vitamin B$_{12}$ import cycle.

---

**Figure A2:** Binding of tempo-vitamin B$_{12}$ with BtuF. **A** Schematic view of BtuF with bound vitamin B$_{12}$ (PDB 1N4A). A DTPA-Gd(III) label was attached to BtuF (at position 138, which is highlighted in ball and stick). **B** Structure of TEMPO-vitamin B$_{12}$, which was synthesized with a protocol adapted from [122]. **C** DEER measured between DTPA-Gd(III) in BtuF and bound TEMPO-vitamin B$_{12}$. **Left** Primary DEER data, **middle**), form factor after background correction and **right**), the obtained distance distribution using Tikhonov regularization with the software DeerAnalysis2011 [87].
Figure A3: Binding of TEMPO-vitamin B$_{12}$ to the outer membrane transporter BtuB. A) Schematic view of BtuB with bound vitamin B$_{12}$ (PDB 1NQH). A NO label (MTSL) was attached to BtuB (at position 188, which is highlighted in ball and stick). B) DEER measured between NO label in BtuB and the bound tempo-vitamin B$_{12}$. Left) Primary DEER data, middle), form factor after background correction and right), obtained distance distribution using Tikhonov regularization with the software DeerAnalysis2011 [87].
Bibliography


List of Publications

Publications during thesis


Other publications


Patent


Conference Presentations

Oral Presentations

1. Mechanism of vitamin $\text{B}_{12}$ transport by the *E. coli* ABC importer BtuCD-F revealed by pulsed EPR spectroscopy. Presented at The 46th Annual International Meeting of the ESR Spectroscopy Group of the Royal Society of Chemistry, University of Warwick, UK, 2013.

Poster Presentations


4. Conformational transitions of the translocation channel in the vitamin B\textsubscript{12} transporter in micelles and liposomes. EUROMAR, Dublin, Ireland 2012.


6. Transmembrane gating in the vitamin B\textsubscript{12} importer. The 46th Annual International Meeting of the ESR Spectroscopy Group of the Royal Society of Chemistry, University of York, UK, 2011.

7. A distinct mechanism of the vitamin B\textsubscript{12} ABC importer BtuCD revealed by Double Electron- Electron Resonance (DEER). 5th EPR Summer School, University of Konstanz, Germany, 2010.
Acknowledgements

The achievements presented in this thesis work were realized through substantial contributions and support from a lot of people.

First of all I would like to thank Prof. Gunnar Jeschke for giving me the great opportunity to do my PhD in his group. Without the financial and more importantly, the scientific support he provided, it would not have been possible to complete this thesis work. It was a fantastic experience to learn from him during my PhD.

I would like to thank Prof. Kaspar P Locher not only for being the co-examiner, but also for making this study possible by providing the required plasmids and the protocols for the protein purification. I deeply appreciate his valuable time for the critical discussions of the results and evaluations of the manuscripts that came out from this study.

I am indebted to Dr. Enrica Bordignon for the time she invested to introduce me to pulse EPR spectroscopy of membrane proteins. As the project leader, her knowledge and expertise have significantly accelerated the implementation of this project. I was very lucky to have her as the co-supervisor and learn directly from her.

I would like to thank Dr. Birke Goetz from Prof. Kaspar P Lochers’s group for the wonderful support in the beginning with establishment of BtuCD purification. Dr. Maxim Yulikov introduced me to selective distance measurements and I would like to thank him for his valuable time.

I would like to particularly thank Rene Tschaggelar and Kristina Comiotto for their great support during my study. Also, I thank Dr. Yevhen Polyhach for the scientific discussions and help and for the support during difficult times.

I thank my lab members Andrin Doll, Kamila Guerin, Sahand Razzaghi and Simon Peter Böhm for the nice company and the friendship during my stay in the group. I would like thank other past and present members of the group for the support they gave me: Dr. Inés Garcia Rubio, Jörg Forrer, Luca Garbuio, Oliver Oberhänsl, Petra Lüders, Remmia Mathews, Thomas Kohn, Tona von Hagens and Udo Kielmann.

Finally I would like thank my friends and family, particularly my wife Remmia Mathews. I deeply appreciate the love, support and the understanding Remmia provided me during this time, without which this work would not have been finished as of now.

Above all, I thank the almighty for providing me with the strength throughout my life!
Curriculum Vitae

Benesh Joseph

Address  
Lilienweg 3  
8952, Schlieren  
Switzerland  
Tel: +41 44 380 4170  
E-Mail: benesh.joseph@phys.chem.ethz.ch

Nationality  
Indian

Date of Birth  
May 29th 1980

Gender  
Male

Education

2009-2013  
Swiss Federal Institute of Technology, Zurich, Switzerland.  
PhD in the group of Prof. Gunnar Jeschke. Topic: Investigation of the  
Vitamin B\textsubscript{12} Translocation Mechanism for the \textit{Escherichia coli} ABC Importer  
BtuCD-F using EPR Spectroscopy.

2006-2008  
Osaka University, Japan  
M.Eng. Biotechnology. Topic: Global metabolic profiling and chemometric  
analysis of metabolome from \textit{Orobanchea minor} seeds during conditioning  
and germination.

2001-2002  
University of Calicut, India  
M.Sc. Microbiology. Topic: Search for spectral signatures in microbe  
fermented Ayurvedic medicines.

1997-2000  
University of Kannur, India  
B.Sc. Microbiology
Work Experience

09.2009 - 05.2013  Research/Teaching Assistant, ETH Zurich, Switzerland
01.2009 - 08.2009  Research Assistant, University of Zurich, Switzerland
09.2005 - 09.2006  Lecturer, University of Calicut, India
09.2004 - 09.2005  UNESCO Postgraduate Researcher, Osaka University, Japan
01.2003 - 06.2004  Lecturer, University of Calicut, India

Awards

2011  Best poster award from British Biophysical Society
2009  UZH|ETHZ Molecular Life Sciences PhD fellowship, Switzerland- declined
2009  Japanese Government Monbukagakusho Scholarship for PhD, declined
2006  Japanese Government Monbukagakusho Scholarship for M.Eng., Japan
2004  UNESCO-Japan fellowship in Biotechnology, Japan
2004  National Eligibility Test (NET) for Lectureship, CSIR, India
1995  Rajyapuraskar for boys scouting from Governor, Govt. of Kerala, India