Conformational Cycle of the Vitamin B$_{12}$ ABC Importer in Liposomes Detected by Double Electron-Electron Resonance (DEER)*#1

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Background: Type II ABC importers transport diverse substrates into the cell.

Results: EPR on BtuCD-F in liposomes shows the response of cytoplasmic gate II during nucleotide cycle in the presence of substrate.

Conclusion: The cytoplasmic gate II closes with substrate and ATP as in the x-ray structure. Substrate can be released after hydrolysis.

Significance: There is new insight into the mechanism of transport in membranes.

Double electron-electron resonance is used here to investigate intermediates of the transport cycle of the *Escherichia coli* vitamin B$_{12}$ ATP-binding cassette importer BtuCD-F. Previously, we showed the ATP-induced opening of the cytoplasmic gate I in TM5 helices, later confirmed by the AMP-PNP-bound BtuCD-F crystal structure. Here, other key residues are analyzed in TM10 helices (positions 307 and 322) and in the cytoplasmic gate II, i.e. the loop between TM2 and TM3 (positions 82 and 85). Without BtuF, binding of ATP induces detectable changes at positions 307 and 85 in BtuCD in liposomes. Together with BtuF, ATP triggers the closure of the cytoplasmic gate II in liposomes (reported by both positions 82 and 85). This forms a sealed cavity in the translocation channel in agreement with the AMP-PNP-BtuCD-F x-ray structure. When vitamin B$_{12}$ and AMP-PNP are simultaneously present, the extent of complex formation is reduced, but the short 82–82 interspin distance detected indicates that the substrate does not affect the closed conformation of this gate. The existence of the BtuCD-F complex under these conditions is verified with spectroscopically orthogonal nitroxide and Gd(III)-based labels. The cytoplasmic gate II remains closed also in the vanadate-trapped state, but it reopens in the ADP-bound state of the complex. Therefore, we suggest that the substrate likely trapped in ATP-BtuCD-F can be released after ATP hydrolysis but before the occluded ADP-bound conformation is reached.

ABC$^2$ transporters are one of the largest families of membrane proteins, mediating the transport of diverse substances across the membrane (1, 2). They consist of two transmembrane domains connected to two nucleotide binding domains (NBDs) that bind and hydrolyze ATP. Bacteria possess both ABC importers and exporters. Based on transmembrane domain architecture, ABC importers are classified into type I and type II (3). The energy-coupling factor transporters constitute another type of ABC importers (4).

Here, we investigate the nucleotide- and substrate-dependent conformational changes of BtuCD, a type II ABC importer mediating uptake of vitamin B$_{12}$ with the help of the substrate-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 (5, 6). Importantly, the BtuCD-F apo x-ray structure showed an occluded translocation channel with no space for vitamin 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 (7) and proven by double electron-electron resonance (DEER) (8) was confirmed by the AMP-PNP (a nonhydrolysable ATP analog)-bound BtuCD-F structure. Crystals were obtained on the ATPase-deficient E159Q mutant stabilized by cysteine cross-linking of the NBDs (supplemental Fig. S1 shows the comparison between the distance simulated on the new structure and the experimental distances published in Ref. 8).

In the AMP-PNP crystal structure, the swing-out motion of the cytoplasmic gate I in TM5 helices was found coupled to the closure of two short loops between TM2 and TM3 (9), named cytoplasmic gate II. 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 AMP-PNP (9). Cross-linking experiments performed on detergent-solubilized transporters confirmed the closure of this gate in the presence of ATP. Interestingly, a recent EPR study suggested the existence of a similar sealed cavity in the transloca-

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§ The abbreviations used are: ABC, ATP-binding cassette; AMP-PNP, β,γ-imi-
doadenosine 5’-triphosphate lithium salt hydrate; DEER, double electron-electron resonance; NBD, nucleotide-binding domain; MTSL, (1-Oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate; LDAO, lauryldimethylamine-N-oxide; DTPA, diethylenetriaminepentacetic acid.
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**Experimental Procedures**

Protein Expression, Purification, and Spin Labeling—Expression and purification of BtuF and BtuCD and the ATPase activity assay were performed as described before (8). Cysteine mutation at position 138 was introduced in His-tagged wild type BtuF containing two native cysteines, which are cross-linked and unavailable for labeling. Cysteine mutations in BtuCD at positions 82, 85, 307, and 322 were introduced on a “Cys-less” plasmid. BtuCD expressed in *E. coli* BL-21 (DE3) Gold cells was extracted and purified in lauryldimethylamine-N-oxide (LDAO). The 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 and sonicated (Sonics vibra-Cell). Following centrifugation, the lysate was centrifuged for 30 min at 40,000 × g and loaded onto a nickel-nitrilotriacetic acid column, pre-washed with the same buffer containing 0.1% LDAO. After loading, 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. Protein was eluted with 50 mM Tris–HCl (pH 7.5) containing 200 mM imidazole, 0.1% LDAO, and 500 mM NaCl and immediately desalted with a HiPrep desalting column (GE Healthcare) into the same buffer without imidazole. Spin labeling was performed with 15–20 μM BtuCD with 40-fold molar excess of MTSL added in four portions in 5-min intervals at room temperature. Unreacted labels were removed with a PD10 desalting column (GE Healthcare). For complex formation, BtuF was added at this stage at 2:1 molar ratio to BtuCD. The functionality of the protein preparation was analyzed with an established ATPase assay protocol as described before (8, 10). Briefly, the ATPase assay reactions were performed at room temperature. Reactions contained 0.01 mg/ml BtuCD or BtuCD–F, in 50 mM Tris–HCl (pH 7.5), 500 mM NaCl, and 0.1% (w/v) LDAO or in 0.1% LDAO and 10 mM MgCl₂. Reactions were started by addition of 2 mM ATP (Sigma), and at various time points, 50 μl were mixed with an equal volume of 12% (w/v) SDS to stop the reaction. A colorimetric assay was used to determine the inorganic phosphate as described before (8, 10).

The levels of expression in *E. coli* for BtuCD were not affected by any of the mutations performed. BtuCD and BtuCD–F spin-labeled at positions 82, 307, and 322 had ATPase activities (Table 1) comparable with those reported for wild type transporter (10) and other spin-labeled mutants (8), whereas labeling at position 85 reduced the activity probably due to the tight location of the spin-labeled side chains in the ATP-bound state. The spin labeling efficiencies varied between

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To allow complex formation directly in proteoliposomes in the absence and presence of BtuF were previously detected for wild type BtuCD in LDAO, and a lower activity around 300–500 nmol Pi min⁻¹ in the absence and presence of BtuF (with or without vitamin B₁₂). The lipidic suspension was centrifuged and the pellet resuspended (without washing step) in about 25 μl buffer; 10% v/v deuterated glycerol was added to the samples before snap-freezing in liquid nitrogen (Fig. 5).

To prepare samples for Gd(III)-NO DEER, reconstituted BtuCD (300–500 μM of 15 or 10 μM BtuCD carrying the nitroxide labels at positions 82 or 168, respectively) was extruded 10 times (400 nm membrane) and frozen and thawed five times with 15 μM vitamin-loaded BtuF-Gd(III)-labeled at position 138 with or without 1 mM AMP-PNP. The lipidic suspension was centrifuged, and the pellet was resuspended in about 25 μl of buffer; 10% v/v deuterated glycerol was added to the samples before snap-freezing in liquid nitrogen (Figs. 5 and 6).

**Interspin Distance Determination—Low temperature EPR spectra to determine short interspin distances (<2 nm) were recorded in EPR quartz capillaries (3 mm outer diameter, sample volume 30 μl) on a continuous wave EPR Bruker spectrometer Elexys E500 at 160 K. The spectra were detected with 100-kHz field modulation, 0.08-milliwatt microwave power, 0.25 millitesla modulation amplitude. Dipolar-broadened EPR powder spectra were fitted with the software DIPFIT (26). The line width parameters of the reference nondipolar-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 supplemental 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 Aₓyz, the interspin distance, and the width of the distance distribution (Gaussian model).

DEER measurements were performed on a homemade or commercial (Bruker ELEXYS-II E580) Q-band spectrometer (34–35 GHz) equipped with a TWTA amplifier (150–200 watts) and a homemade rectangular resonator enabling the insertion of sample tubes with a 3-mm outer diameter (27, 28). 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 eight steps (28). For NO-NO DEER, the ELDOR frequency was set at the maximum of the echo-detected field sweep 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 sweep spectrum for NO, 300 MHz higher than the observer frequency for Gd(III) ions. The back-

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**Table 1**

| Spin-labeled position | ATPase activity | Spin labeling efficiency |
|-----------------------|-----------------|-------------------------|
| 82 (TM2–3 loop)       | BtuCD           | 970                     |
|                       | BtuCD-F         | 1110                    |
| 85 (TM2–3 loop)       | BtuCD           | ND                      |
|                       | BtuCD-F         | ND                      |
| 307 (TM10)            | BtuCD           | 1350                    |
|                       | BtuCD-F         | 1600                    |
| 322 (TM10)            | BtuCD           | 1640                    |
|                       | BtuCD-F         | 2080                    |

The assay used in this study was performed as described previously (8) using three replicates for each measurement. Values in the 1000–1200-nmol Pi min⁻¹ range were found in liposomes (11). ATPase assay reactions were performed as described under “Experimental Procedures.” Deviations due to the reproducibility of the ATPase activity were calculated to be in the 10–20% range. The error in the spin labeling efficiency determination by double integration of the room temperature continuous wave EPR spectra and in the protein concentration in detergent is in the 10% range. The determination of the protein concentration in liposomes is affected by a larger error than in detergent due to difficulties in calculating the reconstitution yield with high precision based on the labeling efficiency calculation. ND means not determined.

56 and 80% for the different mutants. However, even at the lowest labeling efficiencies obtained, only about 10–15% of the dimeric transporters are the respective Cys mutants, and thus the ATPase values are representative of the spin-labeled protein activity. Notably, the distances measured by DEER concern only those transporters carrying two spin labels; the fraction of singly labeled transporter only modifies the modulation depth in the DEER trace but not the obtained distances; the unlabeled proteins are EPR-silent.

Reconstitution in Liposomes—Liposomes containing *E. coli* polar lipid extract and egg l-α-phosphatidylcholine at a 3:1 ratio (w/w) in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl were prepared as described before (8) and stored at −80 °C until use. Before reconstitution, BtuCD and BtuCD-F were concentrated to 20–30 μM, and reconstitution was performed directly from LDAO using an established protocol (11) with minor modifications (8). A 1:1000 molar ratio of BtuCD to lipids was used. Following reconstitution, the liposome suspension was centrifuged down at 170,000 g. The pellet was resuspended in buffer to achieve a spin concentration in the 50–100 μM range. Protein concentration for the ATPase activity assay was calculated using the spin labeling efficiency determined in detergent and the spin concentration measured in proteoliposomes. BtuCD and BtuCD-F were reconstituted in liposomes in the apo-state (absence of nucleotides). The ATP-bound state was prepared by incubating the concentrated BtuCD or BtuCD-F samples (50–100 μM) at 4 °C for 10 min with 1 mM ATP and 50 μM EDTA or with 1 mM AMP-PNP and 2 mM MgCl₂. The ADP state was obtained by incubating the sample at 4 °C for 10 min with 1 mM ADP and 2 mM MgCl₂. For BtuCD it was shown that the reconstitution yields a >90% (11) inside-out orientation of the transporters. This is confirmed by the complete ATP-induced change in the distance distribution at position 85, indicative of accessible NBDs. In the case of BtuCD-F, upon ATP binding we observed a nearly complete change in the distance distributions at position 82, but other sites showed overlap in distances that did not allow drawing conclusions on the precise directionality of reconstitution.

To allow complex formation directly in proteoliposomes in the presence of vitamin B₁₂ and/or nucleotides, reconstituted BtuCD (8–10 μM, 300–500 μl) was extruded 10 times through a 400-nm membrane and afterward frozen and thawed five times with nucleotides and five times with BtuF (with or without vitamin B₁₂). The lipidic suspension was centrifuged and the pellet resuspended (without washing step) in about 25 μl buffer; 10% v/v deuterated glycerol was added to the samples before snap-freezing in liquid nitrogen (Fig. 5).
ground of the normalized DEER primary data \( (V_0/F_0) \) was fitted, and the resulting normalized secondary data \( (F_c/F_0) \) were converted by a model-free Tikhonov regularization to distance distributions with the software DeerAnalysis2011 (29). For clarity, only the obtained distance distributions are shown in Figs. 2–4. The primary and secondary DEER data and all the fits corresponding to the distances presented in the figures are shown in the supplemental material. The simulation of the possible spin label rotamers attached at a position was performed using the Matlab program package MMM based on a rotamer library approach (30).

RESULTS

Response of BtuCD to Nucleotide Cycle—The crystallized BtuCD apo-state shows an outward-facing translocation channel (6), which was also trapped in the crystal of the related HmuUV transporter (32). BtuCD alone has basal ATPase activity in both liposomes and LDAO micelles (11), but it is still unclear how the nucleotide cycle at NBDs is coupled to conformational changes in transmembrane domains. To address this question, we investigated BtuCD in liposomes to obtain distance constraints between spin-labeled pairs in the cytoplasmic gate II, i.e. the loop between TM2 and TM3 and in TM10. The experimental apo- and ADP-Mg\(^{2+}\) distances were found to be very similar (Fig. 2, black and green lines, and supplemental Figs. S2–S5). The mean experimental distances are in agreement with those simulated with MMM (Fig. 2, black dotted lines) on the apo-BtuCD structure (PDB 1L7V). The deviations between the experimental data and the simulations are close to the accuracy achievable with the adopted rotamer library approach (3–3.5 Å) (33) for positions 82 and 85. For position 307 and 322, only part of the experimental distance distribution overlaps with the simulated one, indicating that a fraction of the calculated rotamers may not be populated in BtuCD in liposomes. Previously, we showed that the agreement was found to be good for position 168 in TM5 but poor for position 141 in cytoplasmic gate I (8).

Binding of ATP to the NBDs induces some changes in the translocation channel in BtuCD (Fig. 2, positions 307 and 85) (8), highlighting the BtuF-independent communication from the NBDs to the transmembrane domain. Intriguingly, we could detect an ATP-induced decrease in the 85–85 distance (from 2.2 to 1.6 nm), but not in the 82–82 distance, the latter showing an invariant 3 nm main distance peak accompanied by some distances around 4 nm.

Analyzing the same nucleotide-induced distance changes in LDAO-solubilized transporters highlights the effect of the membrane environment on the distance distributions for particular sites (supplemental Figs. S2–S5), which could be correlated with the known effect on the ATPase activity (11). In LDAO-solubilized BtuCD, for example, the 82–82 distance distribution (supplemental Fig. S2) is found to be very narrow and centered at 1.8 nm (versus 3 nm in liposomes); addition of ATP induces a slight distance decrease between the nitroxide probes. In contrast, the mean 85–85 distance is similar in LDAO and in liposomes in the apo-state, but addition of ATP to the detergent-solubilized BtuCD results in a broad distance distribution from 5 nm down to 1.3 nm, indicative of an ATP-induced close contact between the labeled side chains in the cytoplasmic gate II and a possible ATP-induced protein destabilization (supplemental Fig. S3). For position 307 (supplemental Fig. S4), very similar distances are detected in LDAO and in liposomes, but for position 322 (in TM10 at the C-terminal end of BtuC, supplemental Fig. S5) the mean distance increases to 5 nm in detergent compared with 3.5 nm in liposomes.

Apo-BtuCD-F, Conformation of the Translocation Channel—Fig. 3 shows the DEER data on the cytoplasmic gate II (positions 82 and 85) and on TM10 (positions 307 and 322) obtained on BtuCD-F complexes pre-formed in LDAO detergent (1:2 BtuCD to vitamin B\(_{12}\)-loaded BtuF) and subsequently reconstituted, which leads to a liposome preparation without vitamin B\(_{12}\) (5, 8).

![Conformational changes in BtuCD](image-url)
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Comparison between the experimental distance distributions in the apo-state (Fig. 3, **black**) with those simulated with the software MMM (30) on the BtuCD-F apo-crystal structure (Fig. 3, **black dotted**) shows a very good agreement for the labels in the cytoplasmic gate II (positions 82 and 85). For position 322 in TM10, only part of the simulated distances overlaps with the distance distribution experimentally detected, pointing toward a different arrangement of the rotamers in the BtuCD-F in liposomes with respect to those trapped in the crystal structure.

For position 307, the simulated distances overlap with the range experimentally measured, but due to the tight location of the calculated labels in the crystal (**supplemental Fig. S4I**), the predicted distance distribution is narrower. In this condition, the position of the simulated rotamers is very sensitive to the approximation used in the simulation approach and to the resolution of the underlying structure (30). Overall, the analysis suggests that the distances measured in BtuCD-F in liposomes are consistent with the crystal showing an occluded translocation channel.

Notably, comparison of the interspin distances measured in the BtuCD-F apo-state in LDAO micelles versus liposomes showed a longer and more broadly distributed distance between the cytoplasmic ends of TM10 (position 322, 5 nm in LDAO versus 3.5 nm in liposomes, **supplemental Fig. S5**) and a decreased 82–82 distance in the cytoplasmic gate II (1.7 nm in LDAO versus 2.1 nm in liposomes, **supplemental Fig. S2**), highlighting the influence of the environment on the observed interspin distances.

**ATP Binding to the NBDs Closes the Cytoplasmic Gate II in BtuCD-F in Liposomes**—To verify if the crystallized AMP-PNP-BtuCD-F structure is a good representation of the complex in liposomes in the absence of vitamin B12, we performed a detailed analysis of the experimental distances obtained by EPR and those simulated on the available structure (PDF 4F13). Addition of ATP-EDTA (or AMP-PNP, see **supplemental Fig. S6**) induces detectable changes both in the periplasmic and cytoplasmic ends of TM10 helices and particularly in the cytoplasmic gate II (Fig. 3).

The mean distance between the periplasmic ends of TM10 (positions 307) increases by about 1 nm upon ATP binding (Fig. 3, compare **black** and **red** distributions). This was confirmed by the decreased dipolar broadening in the low temperature continuous wave EPR spectra (see **supplemental Fig. S4H**). Comparison with the distances simulated on the AMP-PNP-BtuCD-F structure (Fig. 3, **red dotted lines**) reveals a discrepancy. The simulated distances show three sharp peaks in the 3.2–4.5 nm range, characteristic of few rotamers populated at the chosen sites (**supplemental Fig. S4J**). To verify if the distances experimentally observed can in principle be in agreement with the positions of the backbone atoms of residues 307 in the crystal, a simulation using all possible rotamers in the MTSLS library and disregarding the neighboring side chains was performed. In this case, a reasonable agreement between simulations and experiments can be reached (**supplemental Fig. S4J, pink distribution**), which allows the conclusion that the distances observed can be reconciled with the structure in that region by assuming different side chain orientations around the labels.

The cytoplasmic ends of TM10 helices (positions 322) from the apo- to the ATP-bound state show only a rearrangement of the bimodal distance distribution toward longer distances (Fig. 3). The longer distance peak is in agreement with the simulated distances.

The spin labels at the cytoplasmic gate II (positions 82 and 85) report a distinct change in interspin distances upon ATP binding to the NBDs. The change in the 82–82 mean distance from 2.1 to 1.6 nm is in full agreement with the closure of the loops seen in the AMP-PNP BtuCD-F structure (Fig. 3, **red straight and dotted lines**). However, a second peak centered at 3–4 nm (highlighted with an asterisk in Fig. 3) appears that was not present in the apo-BtuCD-F sample before the addition of the nucleotides. This fact rules out the possibility that the 3–4 nm distance arises from a fraction of BtuCD-F, which did not interact with the nucleotides. This distance range cannot be reached by changing the rotamer populations at those sites for a fixed backbone conformation, but interestingly, it is characteristic of the ATP-BtuCD conformation (Figs. 2 and 4). This suggests that ATP or AMP-PNP induces partial dissociation of BtuCD-F complexes in liposomes, in agreement with data published by Lewinson et al. (13). A similar effect, although with lower peak resolution (due to distance overlapping), may be indicated in the distance distributions of positions 322 and 307 as well (Fig. 4).

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 (9), also showed a characteristic distance decrease upon ATP binding to BtuCD-F (Fig. 3) at least in a fraction of the transporters in the sample, confirming the results obtained with the reporter label at position 82. The 85–85 distance simulated on the BtuCD-F crystal structure shows close rotamer contacts (distances down to 1 nm, which is the borderline range for the rotamer prediction) (**supplemental Fig. S3, D and G**). In contrast to position 82, the
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overlap between the 85–85 short distance in ATP-BtuCD and ATP-BtuCD-F does not allow us to distinguish the two fractions likely existing in the sample (Fig. 4).

In detergent-solubilized BtuCD-F carrying the reporter labels at position 85, we also detected the ATP-induced closure of the cytoplasmic gate II (distances <1.3 nm), in line with the available cross-linking data (9). This conformational change of the transporter was likely accompanied by protein destabilization as seen by the broad distance distribution up to 5 nm (supplemental Fig. S3). In LDAO, the tight coupling reported by positions 82 in liposomes (Fig. 3) between ATP binding and closure of the cytoplasmic gate II was not detected. In fact, the short 82–82 distance characteristic of the sealed cytoplasmic gate II was already present in the apo-state of BtuCD-F in detergent and was only slightly modified by binding of ATP (supplemental Fig. S2C). This indicates that the loop between TM2-TM3 has a different response to ATP binding in LDAO than in liposomes.

**ADP-BtuCD-F Has an Occluded Translocation Channel—**

The post-hydrolytic ADP-Mg\(^{2+}\)-bound state of BtuCD-F is an intermediate in the vitamin B\(_{12}\) transport cycle, for which no structural information yet exists. Our results in liposomes show that adding ADP-Mg\(^{2+}\) to the BtuCD-F apo-state resulted in interspin distances close to those detected in the apo-state for all positions investigated (supplemental Figs. S2, S4, and S5, green versus black lines). This suggests that following ATP hydrolysis, the translocation channel adopts a conformation similar to that trapped in the BtuCD-F apo structure, the latter showing no space to accommodate vitamin B\(_{12}\).

**Effect of Vitamin B\(_{12}\) on the Conformation of the Translocation Channel—**

It has been shown that vitamin B\(_{12}\) and ATP enhance complex dissociation both in detergent-solubilized and reconstituted transporters (13). However, vitamin B\(_{12}\) could be trapped only in the presence of AMP-PNP (9). Here, we show the effects of vitamin B\(_{12}\) on the conformation of the cytoplasmic gate II, which is responsible for the sealing of the channel, which traps the substrate. As reporter label, we chose position 82, because it shows characteristic and distinguishable distance distributions in the different states (3 nm in BtuCd; 2.1 nm in apo-BtuCD-F, and 1.6 nm in ATP-BtuCD-F, Figs. 2 and 3). In principle, we could monitor both complex dissociation and conformation of the cytoplasmic gate II solely from the 82–82 distance distributions. However, to directly measure complex formation, we additionally determined the distance between a spectroscopically orthogonal Gd(III) label located in BtuF and two nitroxide (NO) labels in the periplasmic region of BtuC (position 168) or in the cytoplasmic gate II (position 82).

For this type of experiment, we prepared samples by adding 1 eq of BtuF (± vitamin B\(_{12}\)) to BtuCD (spin-labeled at position 82) (10 \(\mu\)M) reconstituted in liposomes. After centrifugation of the liposome suspensions, DEER was measured on the pellet resuspended with a minimal volume of buffer. The DEER traces in the apo-state as well as in the ADP state (Fig. 5, A and B) are similar to those obtained with pre-formed BtuCD-F complexes (Fig. 3 and supplemental Fig. S2). A slight amplitude increase in the peak at 3 nm characteristic of BtuCd was observed in the presence of vitamin B\(_{12}\), but the main peak at 2.1 nm (associated with the 82–82 distance in BtuCD-F) was only marginally affected, indicating that vitamin B\(_{12}\) does not perturb the conformation of the cytoplasmic gate II.

In the presence of AMP-PNP and vitamin B\(_{12}\), a more pronounced complex dissociation is expected based on previous findings (13). This is the most interesting state because it is the state in which vitamin B\(_{12}\) could be trapped. Indeed, the 1.6 nm peak intensity (associated with the 82–82 distance in the AMP-PNP-BtuCD-F fraction) decreased (Fig. 5C), and the 3–4 nm peak intensity increased (associated with the 82–82 distance in the AMP-PNP-BtuCD fraction). Interestingly, despite enhanced complex dissociation, the sealed conformation of the translocation channel (1.6-nm 82–82 distance) is maintained in the existing fraction of BtuCD-F complexes in the presence of vitamin B\(_{12}\). An SDS-PAGE performed with the supernatants of the liposome suspensions used for the DEER samples (inset in Fig. 5A) further confirmed these results. In the absence of nucleotides, the band corresponding to the BtuF molecular weight was almost below the detection limit, although a significant amount of BtuF was detected in the supernatant in the presence of AMP-PNP-Mg\(^{2+}\), indicative of decreased complex formation.

Considering the large fraction of AMP-PNP-BtuCD existing under this condition (Fig. 5C), it is important to confirm that the 82–82 distance centered at 1.6 nm actually represents an existing fraction of the AMP-PNP-BtuCD-F complex. To this end, we labeled BtuF (S138C) with maleimido-DTPA-Gd(III) and the periplasmic gate of BtuCD (position 168) with MTSL and performed Gd(III)-NO DEER (34) to monitor BtuF-BtuCD interactions. This experiment would not be very informative if the nitroxide-based MTSL was used for all positions because of overcrowding of distance distributions in triply labeled BtuCD-F. The Gd(III)-NO experiment has already been tested on model systems (35, 36) and soluble proteins (35, 37), and it can be performed selectively without contaminations from NO-NO distance distributions. A distance distribution centered at 4 nm (mean C\(\beta\)-C\(\beta\) distances of 2.9 and 2.7 nm in the apo structure and AMP-PNP-bound structure, respectively) was detected between BtuF and position 168 in BtuCD (Fig. 5D) using Gd(III) as observer spin and the nitroxide as pump spin (34). The modulation depth of the Gd(III)-NO DEER trace gives direct information on the presence and extent of complex formation; the smaller the modulation depth, the smaller the fraction of BtuCD-F complexes in the sample. Although the modulation depth was significantly reduced (as expected), the measured distance between BtuF and BtuCD in the presence of AMP-PNP and vitamin B\(_{12}\) (Fig. 5D) confirmed that some BtuCD-F complexes exist in liposomes under the experimental conditions used.

Furthermore, by 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 B\(_{12}\) and AMP-PNP a Gd(III)-NO distance >6 nm corresponding to the AMP-PNP-BtuCD-F complex (mean C\(\beta\)-C\(\beta\) distance of 7.0 nm in the AMP-PNP crystal structure) as well as the NO-NO distance at 1.6 nm characteristic of the sealed cytoplasmic gate II in AMP-PNP-BtuCD-F (Fig. 6, A and B). By combining our EPR results with the previously published vitamin B\(_{12}\)-trapping experiments (9), it is
highly likely that this fraction of BtuCD–F complex in liposomes harbors the vitamin B₁₂ inside the translocation channel.

We 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 (13). The vanadate-trapped BtuCD–F mimics the intermediate immediately after ATP hydrolysis, with the phosphate group still present. Interestingly, the 82–82 distance distribution measured in this transition state intermediate was almost indistinguishable from that detected in the AMP-PNP state under identical experimental conditions (Fig. 6C).

DISCUSSION

The set of EPR data presented here provides information on the properties of the translocation channel in BtuCD and in particular of the cytoplasmic gate II in different nucleotide states in the presence and absence of BtuF and vitamin B₁₂. The focus of the study is to understand the role of nucleotides and substrate in the formation of a sealed cavity in the channel and in the dissociation of the complex in proteoliposomes.

All EPR experiments used here to draw conclusions on the translocation mechanism were performed in liposomes, mimicking 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 (18, 19, 38), whereas EPR studies on the aspartate transporter Glt Ph revealed that a membrane environment favors conformations different from those observed in detergent micelles (31, 39). Thus, structural differences between detergent and liposome preparations depend on the protein and the type of detergent. This study shows that for some positions in the BtuCD translocation channel, the distance distributions and the extent of distance changes differ in the two environments. Such a change in distance distributions could be correlated with changes in protein dynamics responsible for the known increase in ATPase activity in LDAO (11).
In the absence of BtuF, BtuCD showed some nucleotide-dependent conformational changes in the translocation channel. In particular, ATP induced a close contact between positions 85 in the cytoplasmic gate II, indicating that this region of the protein is highly sensitive to the presence of ATP at the NBDs. Interestingly, we found that the coupling between binding of ATP and closure of the cytoplasmic gate II (positions 82 and 85) was different in the absence and presence of BtuF, especially at position 82 (Figs. 2 and 3).

In the presence of BtuF, the key conformational changes between the apo and the AMP-PNP-BtuCD-F crystal structures are largely validated by the interspin distances changes determined in liposomes in the absence of vitamin B$_{12}$. We can thus conclude that the distances measured on ATP-BtuCD-F in liposomes are in agreement with the AMP-PNP crystal structure (9).

In particular, the interaction between unloaded BtuF with the apo or ADP states of the transporter led to the formation of a stable complex. The BtuCD-F ADP-Mg$^{2+}$-bound state in liposomes was found to be very similar to the apo-conformation (supplemental Figs. S2–S5 green versus black lines). In both states, the majority of the experimental interspin distances are found to be consistent with the occluded translocation channel in the BtuCD-F apo-crystal structure, with no space for a vitamin B$_{12}$ molecule in the channel (5). In particular, cytoplasmic gate II (positions 82 and 85) is open (Fig. 3), and cytoplasmic gate I (position 141 (8)) is closed. Interestingly, the presence of vitamin B$_{12}$ (1:1 to BtuF) in the apo-state or ADP states did not modify the interspin distances in the translocation channel (Fig. 5, A and B).

Upon ATP binding to BtuCD-F in the absence of vitamin B$_{12}$, both ends of TM10 helices slightly rearrange, the cytoplasmic gate I opens (position 141 (8)), and the cytoplasmic gate II closes (82–82 distance of 1.6 nm; 85–85 distance of 1.5 nm, Fig. 3), with the latter being responsible for sealing of the translocation channel, as observed in the AMP-PNP crystal structure (9). We can thus conclude that the sealing of the translocation channel strictly requires ATP but is a substrate-independent event.

In the presence of ATP and vitamin B$_{12}$, a fraction of the protein sample still showed the 82–82 1.6-nm distance, characteristic of the closed cavity in the BtuCD-F channel, indicating that the sealed cavity is not affected by the presence of substrate (Fig. 5C). Another fraction of transporters in the sample showed a distance distribution characteristic of BtuCD alone (Fig. 5C), consistent with the weaker affinity of BtuF to BtuCD under these conditions (13). Using an orthogonal labeling strategy, we could further prove the existence of the fraction of BtuCD-F complexes in the presence of AMP-PNP and vitamin B$_{12}$ (Fig. 5D). Additionally, we could directly correlate in the same sample the existence of BtuCD-F complexes (via BtuF-BtuCD distance determination) to the presence of a sealed cytoplasmic gate II characteristic of AMP-PNP-BtuCD-F (via 82–82 distance determination, see Fig. 6, A and B). Up to now, we could not directly detect the presence of the vitamin B$_{12}$ in the transporter concomitantly to the conformation of its translocation channel. Our attempts to use a spin-labeled vita...
The complex dissociation is enhanced, which would be critical for avoiding the entry of small molecules from the cytoplasm into the corresponding large cavity. After ADP is exchanged with ATP (present in millimolar concentrations in the cytoplasm), the complex dissociation to start another import cycle.

In the presence of vitamin B₁₂, we found the same conformation of the cytoplasmic gate II in the AMP-PNP and in the vanadate-trapped state, which mimics the transition state intermediate. Thus, the time window for the appearance of the putative inward-facing conformation responsible for substrate release is suggested to be after the transition state intermediate but before the ADP-bound state is reached (Fig. 7). In fact, the latter can be considered a post-translocation intermediate, because it is characterized by an occluded translocation channel with the cytoplasmic gate II open (Fig. 3) and the cytoplasmic gate I closed (8), similar to the apo-state of the complex, as discussed above. 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 B₁₂ to be expelled from the channel. Coupling between these two motions may also be critical for avoiding the entry of small molecules from the cytoplasm into the corresponding large cavity. After ADP is exchanged with ATP (present in millimolar concentrations in the cell), the complex dissociation is enhanced, which would allow the next cycle to start with the capture of another vitamin B₁₂ by the BtuF released in the periplasm and its subsequent rebinding to the ATP-BtuCD (Fig. 7).

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. With the crystal structures obtained using detergent-solubilized transporters, it is important to directly verify that they represent the corresponding states of the protein in a membrane environment. Here, we studied several possible intermediates of the transport cycle for BtuCD and BtuCD-F in liposomes and detergent micelles, and we compared them with the available crystal structures. The EPR data obtained with liposome-reconstituted transporters prove that the inward-facing core translocation channel with a sealed cavity as observed in the AMP-PNP-BtuCD-F crystal structure (9) is present in a membrane environment as well. We show that the presence of vitamin B₁₂ does not change the conformation of the sealed cavity and that the substrate could be released after ATP hydrolysis but before the occluded ADP conformation is reached. Despite having a smaller substrate, another type II ABC importer, MolBCA, was also suggested to form a sealed cavity in detergent micelles in the presence of ATP (10). Thus, the ATP-driven closure of the cytoplasmic gate II appears to be a common feature of these two importers. It remains to be elucidated whether other type II importers share a similar substrate translocation mechanism.

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