Transmembrane Gate Movements in the Type II ATP-binding Cassette (ABC) Importer BtuCD-F during Nucleotide Cycle*§

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ATP-binding cassette (ABC) transporters are ubiquitous integral membrane proteins that translocate substrates across cell membranes. The alternating access of their transmembrane domains to opposite sides of the membrane powered by the closure and reopening of the nucleotide binding domains is proposed to drive the translocation events. Despite clear structural similarities, evidence for considerable mechanistic diversity starts to accumulate within the importers subfamily. We present here a detailed study of the gating mechanism of a type II ABC importer, the BtuCD-F vitamin B₁₂ importer from *Escherichia coli*, elucidated by EPR spectroscopy. Distance changes at key positions in the translocation gates in the nucleotide-free, ATP- and ADP-bound conformations of the transporter were measured in detergent micelles and liposomes. The translocation gates of the BtuCD-F complex undergo conformational changes in line with a “two-state” alternating access model. We provide the first direct evidence that binding of ATP drives the gates to an inward-facing conformation, in contrast to type I importers specific for maltose, molybdate, or methionine. Following ATP hydrolysis, the translocation gates restore to an apo-like conformation. In the presence of ATP, an excess of vitamin B₁₂ promotes the reopening of the gates toward the periplasm and the dislodgment of BtuF from the transporter. The EPR data allow a productive translocation cycle of the vitamin B₁₂ transporter to be modeled.

“two-state, alternating access” mechanic model for both ABC exporters and importers (4). 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, and methionine systems) and type II (e.g. vitamin B₁₂, heme, and metal systems) (3). For type I ABC importers 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 (5–8). 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 1 order of magnitude bigger than those imported by the type I (e.g. vitamin B₁₂, 1355 Da; methionine, 141 Da). To date, only two type II importers have been crystallized: the BtuCD vitamin B₁₂ importer from *Escherichia coli* in the presence and absence of the substrate binding protein BtuF (9, 10) and the BtuCD homologous putative metal chelate importer HI1470/1 from *Haemophilus influenzae* (11). The first BtuCD structure 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 ($K_d \sim 10^{-13}$ and $10^{-9}$ M, for BtuCD-F and Hi1470/1–2, respectively) (12) than in type I ($K_d \sim 10^{-7}$ M) (13). 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) (12). 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 (14), and no evidence for substrate-induced changes in affinity was found. All of these observations highlight the mechanistic differences between type I and type II importers.

Probe techniques such as site-directed spin labeling (15) allow for studying conformational transitions during the transport cycle in ABC transporters (16–21). Methanethiosulfonate spin labels (MTSSL) were previously attached in BtuCD to cysteines strategically placed at the cytoplasmic end of TM5 (Ser-141) and in the short periplasmic loop between TM5 and helix 5a (Thr-168) (10, 20), which are key residues in the substrate translocation channel (see Fig. 1). It was shown by 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 analog of ATP) switched the cytoplasmic gate to a highly mobile conformation (20). Although an increase in motility is not generally correlated with an increase in distance between the two spin labels, Goetz et al. (20) 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 Ser-141, Thr-142, and Thr-168, respectively) to quantitatively follow the movement of the cytoplasmic and periplasmic gates during the nucleotide cycle using pulse EPR techniques. In the present work, 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$_{12}$ into the cytoplasm. An EPR-based model of a productive translocation event is provided.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of BtuF and BtuCD**—BtuF was purified as described previously (22). BtuF loaded with vitamin B$_{12}$ was stored at 4 °C up to 3 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 (10). BtuCD was extracted and purified in lauryldimethylamine N-oxide (LDAO) as described previously (9) with some modifications. BtuCD mutants were overexpressed in _E. coli_ BL21(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 min at 40,000 × g and then loaded onto a nickel-nitrilotriacetic acid column, prewashed 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. Protein aggregation was checked by size-exclusion chromatography with a Superdex 200 10/300 column (GE Healthcare).

**Spin Labeling of BtuCD Mutants**—After desalting, BtuCD was concentrated to 15–20 μM with 50-kDa Amicon Ultra Concentrators (Millipore). A 40 fold molar excess of MTSSL was added in 4 batches every 5 min at room temperature while shaking. To prepare BtuCD-F, vitamin B$_{12}$-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 (22). 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.

**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 (22) 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 (22). ATPase activities were measured for all spin-labeled mutants according to Borths et al. (22).

**Sample Preparation for EPR Measurements**—BtuCD and BtuCD-F in LDAO or in liposomes were prepared in the apoprotein (absence of nucleotides). The ATP-bound state was prepared incubating the sample for 5 min 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 min at 37 °C with 1 mM ATP and 2 mM MgCl$_2$ or directly by incubation at 4 °C for 5 min with 1 mM ADP and 2 mM MgCl$_2$. For DEER measurements, 10% (v/v) deuterated glycerol was added to the samples before snap freezing them in liquid nitrogen.

**Continuous Wave and Pulse EPR**—All continuous wave (cw) X-band EPR experiments were performed with a Bruker Elecsys E580 spectrometer equipped with a Bruker Elecsys Super High Sensitive probehead at room temperature or at 160 K using a Bruker N$_2$ flow cryostat. EPR spectra were detected at room temperature in EPR glass capillaries (0.9-mm inner diameter; sample volume, 15 μl) with a 100 kHz field modulation, 2
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milliwatts of microwave power, and a modulation amplitude of 0.15 milliteslas. EPR spectra for interspin distance determination were recorded at 160 K in EPR quartz capillaries (3-mm outer diameter; sample volume, 30 μL) with 100 kHz field modulation, 0.08 milliwatts of microwave power, and a modulation amplitude of 0.25 milliteslas. Fitting of dipolar-broadened EPR powder spectra was performed with the software DIPFIT (23). The line width 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 Elexys 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 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 an outer diameter of 3 mm (24). 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 π/2 and π pulses, with the ELDOR π 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 eight 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 eight 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 (25). 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 (26).

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 (9, 10). Fig. 1 shows the crystal structures of the vitamin B12 importer, with the two gate positions spin labeled in silico with MTSSL based on a rotamer library approach implemented in the software MMM (26). 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.

During the past few years, DEER (also known as pulsed electron electron double resonance) has been widely used as the most sensitive technique to extract distances in the 2–6 nm range (27) on a variety of membrane proteins. For shorter distances (1–2 nm), the analysis must be complemented with line shape fittings of low temperature cw EPR spectra (23).

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 (22), except for 142R1, which showed a 50% activity reduction. Nevertheless, the 142R1 mutant in LDAO was proven to be able to bind ATP and

![Figure 1. BtuCD and BtuCD-F, simulation of interspin distances. A, ribbon representation of the x-ray structures of BtuCD and BtuCD-F (Protein Data Bank codes 1L7V and 2QI9, respectively). The calculated spin label rotamers attached to the engineered cysteines S141C, T142C, and T168C with the software MMM are shown in red and blue ball and stick representation. 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. Dotted gray line, BtuCD; dotted black line, BtuCD-F.](image-url)
ADP, undergoing conformational changes in line with those observed in 141R1. In proteoliposomes, all spin-labeled mutants showed wild type-like ATPase activity, with 141R1 being the most active (supplemental Table S1).

Opening and Closing of 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 the absence of vitamin B12, as observed previously (10). 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 (supplemental Fig. S7).

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 (22). 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...
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distances) was induced upon ATP addition (Fig. 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 (Fig. 2A). ATP binding decreases the interspin distance to <2 nm (Fig. 2A), toward 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 (Fig. 2A, inset in the third panel). The mean distance revealed by line shape analysis (supplemental Fig. S2) is 1.6 nm (Gaussian dotted line in the (r) panel of Fig. 2A), in line with the DEER data. ATP hydrolysis reopens the periplasmic gate, restoring an apo-like conformation, as shown by DEER and cw EPR (supplemental Fig. S2).

In the apo-state, the distance between the two labels at position 141 in the cytoplasmic gate is centered at 1.7 nm (Fig. 2B). ATP binding opens the cytoplasmic gate by 0.7 nm, opposite to the 0.4-nm decrease between the R1 side chains in the periplasmic gate. ATP hydrolysis restores the cytoplasmic gate back to an apo-like conformation. Spin normalized low temperature EPR spectra (inset in the third panel in Fig. 2B) and line shape analysis (supplemental Fig. S2) confirmed the movements observed by DEER.

A similar distance increase was detected also between positions 142 in the cytoplasmic gate. It is worth noting that for positions 142, the distance increase observed upon ATP binding is accompanied by a decrease in mobility in the cw spectra (supplemental Fig. S1). The narrow distance distribution in the apo-state is indicative of spin labels strongly interacting with neighboring amino acids. The ADP-bound state is found to be indistinguishable from the apo-state. In summary, the gates of BtuCD-F are shown to switch synchronously upon ATP binding, with the cytoplasmic gate opening and the periplasmic gate closing, suggesting an inward-facing conformation.

Opening and Closing of Gates in Detergent (LDAO)—All data presented above were obtained in proteoliposomes in the presence of the substrate binding protein BtuF to mimic as closely as possible the physiologically relevant state of the transporter. The question of whether the membrane is necessary for the nucleotide-driven conformational switch was investigated by analyzing the complex solubilized in LDAO micelles. It has been shown that BtuCD or the preformed complex BtuCD-F can switch from the open to the closed state.

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.

BtuF: Nucleotide- and Vitamin-dependent Affinity to BtuCD—It has been shown that BtuCD or the preformed complex BtuCD-F cannot bind vitamin B12 (12, 22). 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 B12) and in LDAO (vitamin B12 to BtuF 1:1 ratio). Excess of ATP or ADP-MgCl2 (up to 5 mM) had no effect on the affinity of BtuF for BtuCD at the micromolar protein concentrations used. Interestingly, a vitamin B12 excess was shown recently to preferentially impair BtuF binding in the ATP-state of the transporter.
We conducted experiments using cw and pulse EPR to monitor the influence of a molar excess of vitamin B₁₂ 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 positions 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.

The room temperature cw EPR spectra of positions 168 in BtuCD and BtuCD-F in LDAO (supplemental Fig. S1 and inset in Fig. 5A) show that binding of BtuF produces a characteristic peak in the low field region due to the reduced mobility of the R₁ side chains (asterisk in the inset in Fig. 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 confirmed that at high vitamin concentrations the periplasmic and cytoplasmic gates adopt a conformation close to the one observed in the ATP-bound state of BtuCD (Fig. 5). In contrast, the same experiments performed in the ADP-state in both mutants showed a reduced dissociation of BtuF (supplemental Fig. 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.

**DISCUSSION**

The present study reveals how the transmembrane gates in the vitamin B₁₂ transporter BtuCD-F respond to the presence of BtuF, substrate, and nucleotides. The vitamin B₁₂ 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 gate 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 (Protein Data Bank code 1L7V) with MMM (26) and experimental distances detected in LDAO in the apo-state is presented in supplemental Fig. S5. The broad experimental distance distribution between positions 168 is in line with the simulation (supplemental Fig. 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 as described in A performed in the cytoplasmic gate (142–142). The arrows indicate the reappearance of the BtuCD-like distances in the ATP-state at high vitamin concentration. All DEER traces were recorded at Q band.

for the discrepancies are the following: (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, and (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 (supplemental Table S1).

The simulations performed with MMM on the BtuCD-F structure (Protein Data Bank code 2QI9) (supplemental Fig. 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 the simulated rotamers yielding the distance experimentally detected (black rotamers in supplemental Fig. S5C) are under-represented 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 do the in vitro observations correlate 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 (28), the cytoplasmic membrane bound TonB (29), the periplasmic substrate binding protein BtuF (30), and the transmembrane associated BtuCD (31). 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 (32), 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 that is still under investigation. BtuB is expressed at ~200–500 copies per cell (33), and it is post-transcriptionally regulated through alternate RNA structures induced by the binding of adocobalamine to the 5′-UTR region of the mRNA (34). Unlike genes of other import systems, btuCD and btuF are transcribed independently. Both BtuCD (35) and BtuF (28, 30, 36) are expressed at very low levels, and their expression is not regulated by vitamin B₁₂ (35, 37). Although a quantitative analysis of the amount of BtuCD and BtuF in the cell has not yet been performed, about three vitamin B₁₂ binding sites per cell were reported in the periplasm of *E. coli* (28), which are potentially enough for the observed rate of vitamin B₁₂ import (38). Considering the extreme stability of the BtuCD-F complex (12), essentially all of the BtuF in the cell will be in complex with BtuCD. Hence, the

**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 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. Colors are as described in the legends to Figs. 2 and 3.

**FIGURE 5.** *Effect of vitamin B₁₂ 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 (gray), with 1:1 (black) and 1:50 (violet) molar ratio of BtuF to vitamin B₁₂. The characteristic immobile component in BtuCD-F (indicated by an asterisk) shows the interaction between BtuCD and BtuF. The arrow indicates the decrease of the immobile component at high vitamin concentrations. B, the interaction between BtuCD and BtuF. The arrows indicate the reappearance of the BtuCD-like distances in the ATP-state at high vitamin concentration. All DEER traces were recorded at Q band.

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few copies of vitamin $B_{12}$ binding protein detected by White et al. (28) may reflect the fraction of the free BtuF in the periplasm.

The scarce vitamin $B_{12}$ encountered by the bacteria in the milieu may be concentrated in the periplasm by the action of the high affinity BtuB and TonB system (39). 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 (Fig. 5 and supplemental 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 vivo. Concomitantly to the BtuF release, ATP is hydrolyzed, and the gates adopt the more flexible conformation as observed in BtuCD (Fig. 4). The released BtuF can bind the available substrate preferentially reforming the complex with BtuCD in the apo- or ADP-state (Fig. 5 and Lewinson et al. (12)), 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 (22). Although 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 (10) further supports this model. 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 (12). 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 (supplemental Table S1). Considering this high basal ATPase rate and lack of any asymmetry at the NBDs upon BtuF binding (10), it is conceivable that both ATPs are hydrolyzed simultaneously. Based on the above considerations and the EPR data, which are schematically represented in Fig. 6A, we detail a model for a productive vitamin $B_{12}$ transport cycle mediated by BtuCD-F in the cell (Fig. 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 BtuCD-F, the ATP-induced dimerization of the NBDs opens the translocation pathway toward the cytoplasm. A mechanistic implication of these observations is that the TMDs in BtuCD-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 (9). 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 toward the cytoplasm. The EPR data indeed suggest that binding of ATP opens the translocation pathway of BtuCD-F toward 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 a subject of further investigation.

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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, 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 (1LTV), forming the BtuCD-F complex (model superimposed to Protein Data Bank code 2Q99). 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.
