EPR Spectroscopy of MolB$_2$C$_2$-A Reveals Mechanism of Transport for a Bacterial Type II Molybdate Importer$^{[S]}$*

Received for publication, May 7, 2013, and in revised form, May 19, 2013. Published, JBC Papers in Press, May 24, 2013, DOI 10.1074/jbc.M113.483495

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**Background:** ABC importers utilize the energy of ATP hydrolysis to transport substrates into the cell.

**Results:** EPR studies show how three transmembrane gates of MolB$_2$C$_2$-A work in concert to allow substrate to enter the cytoplasm.

**Conclusion:** The movements of these gates indicate an opening and closing limited to small substrates.

**Significance:** This model sheds light on how small nutrients are transported across the membrane.

In bacteria, ATP-binding cassette (ABC) transporters are vital for the uptake of nutrients and cofactors. Based on differences in structure and activity, ABC importers are divided into two types. Type I transporters have been well studied and employ a tightly regulated alternating access mechanism. Less is known about Type II importers, but much of what we do know has been observed in studies of the vitamin B$_{12}$ importer BtuCD$_{2}$. MolB$_2$C$_2$ (formally known as H11470/71) is also a Type II importer, but its substrate, molybdate, is $\sim$10-fold smaller than vitamin B$_{12}$. To understand mechanistic differences among Type II importers, we focused our studies on MolBC, for which alternative conformations may be required to transport its relatively small substrate. To investigate the mechanism of MolBC, we employed disulfide cross-linking and EPR spectroscopy. From these studies, we found that nucleotide binding is coupled to a conformational shift at the periplasmic gate. Unlike the larger conformational changes in BtuCD-F, this shift in MolBC-A is akin to unlocking a swinging door: allowing just enough space for molybdate to slip into the cell. The lower cytoplasmic gate, identified in BtuCD-F as “gate I,” remains open throughout the MolBC-A mechanism, and cytoplasmic gate II closes in the presence of nucleotide. Combining our results, we propose a peristaltic mechanism for MolBC-A, which gives new insight in the transport of small substrates by a Type II importer.

ATP-binding cassette (ABC)$^4$ transporters, one of the largest families of transmembrane proteins, are divided into exporters and importers (1–3). Exporters, found in all domains of life, transport substrates ranging from lipids and peptides to antibiotics and chemotherapeutic drugs (4, 5). Importers, found primarily in bacteria, play vital roles in metabolite and cofactor uptake (6). This study focuses on MolB$_2$C$_2$-A, a molybdate importer found in the opportunistic pathogen Haemophilus influenzae. Despite diversity in substrate and organism, ABC exporters and importers are thought to have a common mechanism of transport, the alternating access mechanism (6–10).

Much of what we know about the ABC import mechanism is based on the detailed studies of Type I importers (8, 11). For this class of importers, nucleotide binding and hydrolysis at the nucleotide-binding domains (NBDs) drive the transmembrane domains (TMDs) to alternate between inward- and outward-facing conformations (6, 12). These conformational changes allow substrates to be taken up from one side of a lipid bilayer and released on the other side. For ABC importers, the role of substrate specificity has been delegated to a periplasmic binding protein (PBP), which scavenges substrate from the periplasm and delivers it to the transporter (13–16).

Most of our understanding of Type II ABC importers has been gained in studies of the vitamin B$_{12}$ importer BtuCD. This large-substrate Type II importer employs a peristaltic transport mechanism (17). Based on EPR studies and the recently elucidated structure (17, 18), the mechanism of BtuCD involves three gates (one periplasmic gate and two cytoplasmic gates) that accept vitamin B$_{12}$ from the PBP into a translocation chamber. Substrate acceptance appears to be in the transition from the outward-facing nucleotide-free state (19) to the nucleotide-bound state (17).

$^*$This work was supported by in part by National Science Foundation Grant MCB1218782. DEER instrumentation was funded by National Institutes of Health Grant S10 RR022422. DEER spectroscopy was carried out at the National Biomedical EPR Center, which is supported by National Institutes of Health Grant P41 EB001980.

$^+$Supported in part by National Institutes of Health Molecular Biophysics Training Grant T32 GM08382 from NIGMS.

$^+$Supported by National Institutes of Health Grant GM070515.

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$^+$The abbreviations used are: ABC, ATP-binding cassette; NBD, nucleotide-binding domain; TMD, transmembrane domain; PBP, periplasmic binding protein; MTSL, S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrryl-3-ylmethyl) methanesulfonothioate; BeF$_2$, beryllium fluoride; CW, continuous wave; AMP-PNP, adenosine 5’-(β,γ-imido)triphosphate; DEER, double electron-electron resonance.

*This article was selected as a Paper of the Week. This article contains supplemental “Materials and Methods,” Figs. 1 and 2, and Tables 1 and 2.

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Type I and II importers differ both mechanistically and structurally. For the Type I importer MalFGK$_2$, ATP can be hydrolyzed with high efficiency only in the presence of substrate-loaded PBP (20, 21). Many Type I importers contain a C-terminal regulatory domain that prevents ATP hydrolysis when bound to substrate (22). In contrast, MolBC and BtuCD can hydrolyze ATP in the absence of PBP or substrate in vitro (23) and contain no known regulatory domain. Like BtuC$_2$, MolB$_2$ (the TMD homodimer) is composed of 20 transmembrane helices as opposed to the 10–12 helices commonly found in Type I transporters. The binding proteins MolA (16) and BtuF (14) (for MolBC and BtuCD, respectively) fall in the same structural groups: class III and cluster A (13, 24). These PBPs do not undergo significant conformational change upon substrate binding, differing from binding proteins known to associate with Type I transporters (24).

To determine whether the BtuCD-F system of transport applies to a small-substrate Type II importer, in this study, we focused on the molybdate importer MolB$_2C_2$ and the cognate PBP MolA (16, 25). MolA was found to bind both molybdate and the structurally similar oxyanion tungstate (16). Because molybdate is a substrate shared between a Type I importer (ModBC-A) and a Type II importer (MolBC-A), we have the opportunity to focus on one of two molybdate transport systems present in the organism _H. influenzae_. The results of our investigation suggest that MolB$_2$C$_2$ utilizes a peristaltic pumping mechanism similar to BtuCD-F, but differences can be found at two of the three gates.

### EXPERIMENTAL PROCEDURES

**Mutagenesis**—The Stratagene QuikChange kit was used to generate the following MolB mutations: N89C, L91C, N93C, I151C, L148C, I171C, D173C, I178C, S180C, V182C, and L185C. Mutants were confirmed by DNA sequencing.

**Purification of MolA and Mutant MolB**—MolA-binding protein was cloned and purified as described by Tirado-Lee et al. (16). MolBC was expressed and purified as described (25) with slight modifications for spin labeling. For spin-labeled samples, protein-loaded nickel-nitrilotriacetic acid resin was washed on the nickel column, eluted, and dialyzed before concentration to 10–12 helices commonly found in Type I transporters. The binding proteins MolA (16) and BtuF (14) (for MolBC and BtuCD, respectively) fall in the same structural groups: class III and cluster A (13, 24). These PBPs do not undergo significant conformational change upon substrate binding, differing from binding proteins known to associate with Type I transporters (24).

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**Selecting Disulfide Cross-linking**—In brief, MolBC cross-linking mutants were purified and concentrated under reducing conditions. In the presence of 20 mM 2-mercaptoethanol, ligands (MolA and/or beryllium fluoride (BeF$_2$) + ATP-Mg$^{2+}$) were incubated with each mutant. BeF$_2$ trapping was required to maintain MolBC in the nucleotide-bound state throughout the ensuing incubations and dialyses. 2-Mercaptoethanol was removed via dialysis. Cross-linking was promoted by the addition of 0.5 mM CuCl$_2$, following by quenching with 1 mM N-ethylmaleimide. Excess copper and N-ethylmaleimide were removed by dialysis. Final samples were analyzed for cross-linking by SDS-polyacrylamide gel shift and ATP hydrolysis activity by enzyme-linked inorganic phosphate assay (Cytoskeleton, Inc.).

**Reconstitution of Transporter into Proteoliposomes**—Phosphatidylcholine lipid extract (Sigma P5638) was dissolved in reconstitution buffer (25 mM Tris and 100 mM NaCl) to a concentration of 50 mg/ml and then clarified and destabilized with 40 mM sodium cholate (Sigma). MolBC was mixed with cholate-destabilized phosphatidylcholine solution in a 1:10 (w/w) ratio. Washed and dried Bio-Beads (Bio-Rad) were added to the phosphatidylcholine, cholate, and protein solution, increasing the sample volume by at least by 50%. After 12 h of incubation, the Bio-Beads were removed, and the sample was diluted ~70 times in reconstitution buffer and centrifuged at 150,000 × g. Proteoliposomes were resuspended in reconstitution buffer.

**Continuous Wave EPR Spectroscopy**—Continuous wave (CW) EPR spectra were measured as described previously (21). The scan width of all spectra was 250 G, and the spectra presented were cropped to the values stated in the figure legends. The ligands used for CW-EPR spectroscopy were as follows: MolA at 2-fold molar excess over MolBC; 10 mM ATP + 1.5 mM EDTA; and 16.5 mM MgCl$_2$. Ligands were serially added to recovered protein samples and then incubated for at least 10 min (60 min for MolA) before being resuspended. Unlike BeF$_2$ trapping, the use of ATP-EDTA allowed the nucleotide to be hydrolyzed for subsequent analysis. ATP-EDTA and the nucleotide analog Mg-AMP-PNP had similar effects on CW-EPR spectra. Mg-AMP-PNP was preferred in studies that did not require hydrolysis, such as preliminary CW-EPR samples and double electron-electron resonance (DEER)-EPR. Given the known activity for each mutant (supplemental Table 1), a 10-min incubation after the addition of Mg$^{2+}$ allowed ample time for each sample to hydrolyze the given amount of ATP and reach an equilibrium (post-hydrolysis) state, where ADP-bound MolBC dominates the transporter population. CW-EPR spectra were collected at room temperature (296 K).

For spectra showing coupled spins, Gaussian-fitted distance distributions were calculated using the Short Distances program written by Altenbach et al. (26). The fitting program was set to calculate the optimal fit and was not limited by a requirement that all fitting peaks sum to 100% of spin in the sample. When needed for distance calculation of a given mutant with particular ligands, a pseudo sum-of-singles spectrum was measured for the same mutant with the same ligands. The sum-of-singles CW-EPR spectrum was determined by analyzing a sample labeled with a 3:1 ratio of diamagnetic acetoxy-MTSL to paramagnetic MTSL. The low residual and $\chi^2$ values indicate good fitting of the simulated CW-EPR spectra to the observed spectra (supplemental Fig. 2, B and D). The 2011 version of MMM was used to predict spin coupling distances from crystal structures (27).

All protein structure graphics were made with PyMOL (DeLano Scientific, San Carlos, CA). Spectra and charts were graphed using Grapher 9 software (Golden Software Inc., Golden, CO).
Transport Mechanism of Type II Importer

RESULTS

To study the nucleotide- and PBP-induced helical rearrangements of MolBC-A, we utilized site-directed spin labeling EPR spectroscopy and disulfide cross-linking in a detergent environment. Both techniques employ cysteine mutations at specific sites along or near the MolBC translocation pathway, which is made up of the core helices and loops around TM5 and TM5a (Fig. 1A).

All MolBC mutants, including spin-labeled EPR spectroscopy mutants, were assayed to ensure their ability to bind and hydrolyze nucleotide in a detergent and lipid environment. Activities in detergent (supplemental Table 1) and lipid (supplemental Table 2) are comparable to published activity levels of BuCD (23). As expected from studies with BuCD, a notable decrease in activity was observed upon reconstitution. Size exclusion chromatography was used to ensure that unloaded MolA (PBP) and MolBC formed a complex in the absence and exclusion chromatography was used to ensure that unloaded MolA and MolBC. The addition of MolA to TM5 and TM5a (Fig. 1A).

For the conditions tested, the addition of molybdate enhances the dissociation of MolA and MolBC. The addition of MolA to concentrations sufficient to overcome this low affinity would result in excessive dilution of MolBC. Moreover, the fast dissociation would make it difficult to test a homogeneous population of MolBC-A in the presence of nucleotide.

Nucleotide Binding Unlocks the Periplasmic Gate—We began our study of the periplasmic gate with the two TM5a helices that occlude the translocation pathway. To understand how these helices rearrange themselves in the presence of nucleotide and PBP, we independently spin-labeled TM5a at two sites, S180C and L185C, and the loop proceeding TM5a at D173C (Fig. 1A).

With the periplasmic gate spin-labeled, CW spectra were recorded in the absence of nucleotide (apo) and then with ATP (plus excess EDTA to remove magnesium). The latter spectra represent the state with nucleotide bound but not hydrolyzed. At residues S180C, L185C, and D173C, CW-EPR spectroscopy showed an increase in spin label mobility in the presence of ATP (Fig. 1B). With the addition of MgCl₂, ATP was hydrolyzed (Fig. 1C), and the mobility at all three sites returned to an apo-like state as demonstrated by the similarities between the apo and ATP + MgCl₂ spectra. The nucleotide-dependent increase in mobility at all three sites can be attributed to a conformational change at TM5a. Specifically, increased mobility could represent additional flexibility (or conformational substates) allowed in TM5a and the adjacent loop. It is also possible that the mobility changes could represent a subtle rearrangement of helices at the periplasmic gate, which removes tertiary contacts at all three spin-labeled sites. DEER-EPR data of TM5a, probed at S180C, further suggest that this mobility shift or helical rearrangement at the periplasmic gate could be due to a very subtle nucleotide-dependent separation of TM5a (supplemental Fig. 1B).

To understand the effects of both MolA and nucleotide at the periplasmic gate, L185C and S180C were analyzed by CW-EPR spectroscopy (size exclusion chromatography showed that spin labeling at D173C prevented MolA binding to MolBC). In the presence of MolA, mobility at L185C was effectively increased upon binding ATP (Fig. 2B), but when the transporter was allowed to hydrolyze ATP-Mg, mobility returned to an apo-like state. Further studies are needed to determine how these helices rearrange themselves in the presence of nucleotide and PBP, we independently spin-labeled TM5a at two sites, S180C and L185C, and the loop proceeding TM5a at D173C (Fig. 1A).

Figure 1. CW-EPR spectroscopy of TM5a residues showing nucleotide-dependent mobility changes at the periplasmic gate. A, MolB residues S180C, L185C, and D173C (shown as red sticks) were spin-labeled with MTSL. TM5 and TM5a are colored cyan or purple according to the TMD monomer. B, CW-EPR spectra for S180C + MTSL (100-G scan width), L185C + MTSL (150 G), and D173C + MTSL (100G). Blue lines, apo state; red lines, ATP-bound state. Mobile and immobile components are identified for the S180C spectra. Peak broadening (induced by close proximity between spin label pairs) is identified for L185C, note that the amount of broadening is the same for all L185C spectra. C, post-hydrolysis spectra (green) of both mutants overlaid with the respective apo spectra. Overlaid spectra have been normalized for equal spin (normalized double integration values).

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The proximity of TM5a V182C and L178C to the respective sites on the adjacent TMD subunit was confirmed by selective disulfide cross-linking. In the apo form and in the presence of MolA, the residues at the periplasmic gate efficiently cross-linked (Fig. 3B). However, when trapped in the ATP-bound state with BeF₂ and ADP-Mg²⁺, less cross-linking was observed at both sites. Furthermore, when L178C or V182C cross-linked, there was a proportional decrease in the rate of ATP turnover, except for the BeF₂-treated samples (Fig. 3C). Cross-linking and activity results with the apo and MolA-bound samples indicate that NBD activity is directly coupled to a conformational shift at the periplasmic gate. In this ATP-dependent change, the occlusion at TM5a unlocks and is free to open, which disrupts disulfide cross-linking. This subtle unlocking of the periplasmic gate represents the open state that may be permeable to substrate.

Because both TM5 and TM5a make up the translocation pathway, we were curious if the periplasmic side of TM5 also undergoes rearrangement during the transport mechanism of MolBC-A. DEER-EPR spectroscopy was used to measure the distances between spin labels at I171C (supplemental Fig. 1C), where there is a slight decrease in the inter-spin distance in the presence of the non-hydrolyzable nucleotide Mg-AMP-PNP. Because the periplasmic side of TM5 is separated by >1 nm, a larger conformational change at this site would be needed to impact the translocation mechanism. These findings indicate that the periplasmic gate is limited to TM5a and not TM5.

FIGURE 3. Effect of ligand on disulfide cross-linking at the periplasmic gate and cytoplasmic gate. A, two residues at the periplasmic gate (Leu-178 and Val-182) and one residue at the cytoplasmic side of TM5 (Leu-148) are shown as red sticks. Residues mutated to cysteines formed a disulfide cross-link across the translocation pathway. B, three different conditions (apo, MolA-bound, and BeF₂/ATP-trapped) were tested for cross-linking at the three sites relative to a Cys-less control. Cross-linking was observed by monitoring the gel shift from monomeric TMD (30 kDa) to dimeric TMD (60 kDa). The bottom band at 24 kDa is the NBD, and the PBP (MolA) is 39 kDa. C, cross-linking was quantified by image analysis (Image) software of the SDS-polyacrylamide gel in B. In-gel dimer formation of Cys-less MolB was likewise quantified and used to correct the cross-linking values of the mutants. For better comparison with cross-linking percentages, the specific activity of each mutant was calculated as a percent of Cys-less specific activity and subtracted from 100 to give the percent inhibition. Error bars were calculated from the standard error of specific activity measurements (n = 3).
Transport Mechanism of Type II Importer

Characterization of MolBC-A Cytoplasmic Gate I—"Cytoplasmic gate I" identified in the apo-BtuCD structure (19) is represented in MolBC by the cytoplasmic side of TM5. To determine the conformational changes at gate I, nearby sites were probed via EPR spectroscopy. CW-EPR allows us to quantify spin coupling when the inter-spin distance is 0.8–1.8 nm; coupling at greater distances cannot be measured with this technique, but the percent of uncoupled spins can be approximated during analysis. A non-cross-linking site along TM5, I151C (Fig. 4A), showed significant spin coupling in the apo state, suggesting a close proximity of spin labels (three distances were calculated: 20.9% at 0.6 nm, 20.6% at 0.9 nm, and 13.3% at 1.3 nm) (supplemental Fig. 2A). From the crystal structure, the inter-spin distance at Ile-151 was predicted to be 0.7 ± 0.3 nm. The addition of ATP and EDTA resulted in a loss of spin coupling, demonstrated by the diminished spectral broadening in the ATP-bound sample relative to the apo sample (Fig. 4B) and the increase in non-interacting spin from 34 to 107% (supplemental Fig. 2A). Non-interacting spin in the apo sample is likely due to incomplete spin labeling. Hydrolysis of ATP-Mg returned some population of spin coupling to an apo-like state (Fig. 4C). These results suggest that the cytoplasmic side of TM5 opens widely upon ATP binding and returns to an apo-like state after ATP-Mg hydrolysis.

MolA did not appear to have an effect on the mobility or coupling of MolBC at I151C in the apo state (Fig. 4D). Whether ATP was added to the MolBC-A complex or MolA was added to ATP-bound MolBC, the same widely separated TM5 conformation at the cytoplasmic gate was obtained (Fig. 4E). In the presence or absence of MolA, the post-hydrolysis state was similar to the apo state (Fig. 4F).

The crystal structure of apo-MolBC shows cytoplasmic gate I in a slightly open conformation (Fig. 3A), which is supported by EPR spectroscopy in the apo state. These results were further confirmed with selective cross-linking experiments of L148C. Leu-148 can be found at the cytoplasmic side of TM5 (Fig. 3A). Compared with sites at the periplasmic gate, L148C shows minimal cross-linking in the apo and MolA-bound states (Fig. 2B and C). When trapped with BeF3−, there is still minimal cross-linking at L148C. These results suggest that the cytoplasmic side of TM5 may be too far apart to efficiently form a disulfide bond. Through these studies, we have shown that cytoplasmic gate I moves from a partially open conformation to a widely separated conformation.

Characterization of MolBC-A Cytoplasmic Gate II—As the cytoplasmic side of TM5 swings out from a partially open state to a widely separated state, the space forming the translocation pathway will increase in size. It is possible that another helix or loop surrounding the translocation pathway may be drawn into this space and form a second cytoplasmic gate, as is seen in BtuCD-F.

To explore the possibility of a second cytoplasmic gate in MolBC, residues of the loop connecting TM2 and TM3 were tested for changes in cross-linking and EPR spin coupling. When spin-labeled at N93C (Fig. 5A) and analyzed by CW-EPR spectroscopy (Fig. 5B), three distances could be observed in apo-MolBC, with the majority of spin (48.1%) centered 0.9 nm (supplemental Fig. 2C). This result fits the distance predicted from the apo crystal structure: 0.9 ± 0.2 nm. Upon ATP binding, the greater part of the spin shifted to 0.6 nm (53.7%) (supplemental Fig. 2C). When ATP hydrolysis was enabled by the addition of MgCl2, the distribution of distances at N93C + MTSL returned to an apo-like state (Fig. 5C). The same conformational changes were observed in the presence of MolA (supplemental Fig. 2C). Throughout the nucleotide cycle, 22–31% of spin remains non-interacting, which is likely due to incomplete spin labeling. These results suggest that the proximity of spin label at N93C narrows when in the ATP-bound state and support the presence of a second cytoplasmic gate in MolBC.

To verify that the loop between TM2 and TM3 could form a closed conformation, three sites along the loop were tested for disulfide cross-linking. In all four conditions tested (apo, MolA-bound, BeF3−/ADP-Mg2+−trapped, and MolA/BeF3−/ADP-Mg2+−trapped), MolBC L91C formed disulfide bonds (Fig. 5D), which is directly comparable to cross-linking of the homologous BtuC mutant L85C (17). The flanking sites N89C and N93C formed disulfide cross-links with or without MolA, but only when trapped in a nucleotide-bound state. These results suggest that it is possible to form a closed conformation at gate II, especially for MolBC in the nucleotide-bound state.
Transport Mechanism of Type II Importer

Details of the mechanistic differences within the family of Type II transporters continue to unfold. From our results, it is immediately clear that the mechanism of MolBC-A has diverged from other ABC transporters, although it bears some similarity to BtuCD-F. The mechanism of BtuCD involves three gates (one periplasmic gate and two cytoplasmic gates) that accept vitamin B12 from PBP into a translocation chamber (29). As expected from similarities in structure and activity, we found that MolBC-A (25) and BtuCD-F (23, 30) share a similar conformational change at the occluding TM5a helices. When cytoplasmic gate II is closed, and binding protein caps the translocation chamber (29), forcing substrate into the translocation pathway (State 1) (Fig. 6C). We speculate that a translocation chamber is formed when cytoplasmic gate II is closed, and binding protein caps the unlocked periplasmic gate (State 3). After ATP hydrolysis, the periplasmic gate locks, and the cytoplasmic gates are open (State 1) (Fig. 6A). In the absence of nucleotide, the periplasmic gate is locked (closed), and the cytoplasmic gates are open (State 1) (Fig. 6B). When ATP binds, the NBDs close around nucleotide. This classical conformational change of the NBDs rearranges the TMD helices and permits substrate to permeate the TM5a occlusion. The mechanistic differences between these two Type II transporters is first apparent at the periplasmic gate, the point of entry for substrates. Based on the crystal structure, TM5a would need to shift during the transport of substrate as it is the point of occlusion in the translocation pathway. As anticipated, the CW-EPR spectroscopy and disulfide cross-linking experiments presented here show that nucleotide binding induces a subtle conformational change at the occluding TM5a helices. This conformational change loosens ternary contacts with adjacent helices and perhaps allows TM5a to assume a more mobile state. Either interpretation of the data may indicate the substrate-permeable (unlocked) state of the periplasmic gate. Moreover, when sites at the periplasmic gate are cross-linked, ATP turnover is inhibited, suggesting that the periplasmic and periplasmic gate are strongly coupled.

Unlike BtuCD, movement at the periplasmic gate is much more subtle, with the shift of TM5a likely to be the only change at the periplasmic gate that impacts the transport mechanism. In other words, the periplasmic gate in MolBC appears to be smaller and offers a more narrow opening than that in BtuCD. However, unlocking of the periplasmic gate is certainly driven by nucleotide binding and hydrolysis.

Although gate I (at the cytoplasmic end of TM5) undergoes significant conformational changes during the nucleotide cycle, it does not appear to assume a closed conformation. In MolBC, cytoplasmic gating appears to rely on gate II at the loop between TM2 and TM3. Cross-linking studies suggest that a closed conformation at gate II is accessible throughout the nucleotide cycle. With a distinct narrowing of inter-spin distance observed via CW-EPR spectroscopy, we conclude that the nucleotide-bound state promotes closure at gate II. This conclusion is supported by our EPR spectroscopy studies of gate I, where TM5 helices appear close together in the apo state (as in the crystal structure) yet widely separated in the presence of nucleotide. Given the arrangement of helices at the cytoplasmic side of the translocation pathway, when gate II is closed, gate I may be forced to a more open conformation.

Our in-detergent results show what conformational movements are possible given the inherent folding of MolBC. Combining these results, we propose the mechanism shown in Fig. 6A. In the absence of nucleotide, the periplasmic gate is locked (closed), and the cytoplasmic gates are open (State 1) (Fig. 6B). When ATP binds, the NBDs close around nucleotide. This classical conformational change of the NBDs rearranges the TMD helices of MolBC, which allows the periplasmic gate to unlock and permits substrate to slip into the translocation pathway (State 2). Concurrently, when ATP is bound, cytoplasmic gate II closes, and gate I is forced to a widely separated conformation (Fig. 6C). We speculate that a translocation chamber is formed when cytoplasmic gate II is closed, and binding protein caps the unlocked periplasmic gate (State 3). After ATP hydrolysis, the periplasmic gate locks, and the cytoplasmic gates assume an apo-like partially open state (State 4).

As expected from similarities in structure and activity, we found that MolBC-A (25) and BtuCD-F (23, 30) share a similar mechanism, but this work also highlighted notable differences between the small- and large-substrate transporters. For MolBC-A, changes at the periplasmic gate are very limited, represented by a narrow opening of the occlusion or perhaps unlocking of a mobile gate. In MolBC-A, the small size of molybdate could allow for this subtle unlocking (opening) of the periplasmic gate, as this is all that is needed for substrate to permeate the TM5a occlusion.
The limited opening and locking mechanism found in MolBC could explain mechanistic differences between the Type II transporters at cytoplasmic gate I. In the apo state, the periplasmic gate of MolBC is closed, so cytoplasmic gate I does not need to protect the transporter from channel formation, as it does in apo-BtuCD. In the nucleotide-bound state, gate II is closed and thus prevents gate I from closing.

Relative to Type II importers, it appears that MolBC has adopted a more limited conformational shift to accept substrate from binding protein. This contrasts with the peristaltic means of transport observed in BtuCD-F (17), where the TMD helices of the Type II importer wrap around and isolate substrate upon acceptance from PBP. The correlation between size of the translocation pathway and cognate substrate is emphasized in the recent structure of the Type II heme transporter HmuUV from *Yersinia pestis*, where the arrangement of helices allows for a smaller translocation pathway (31). Compared with vitamin B_{12}, BtuCD, the periplasmic gate of HmuUV is notably smaller. This makes sense because the size of cobalamin is twice the size of heme (31). Studies with other Type II importers will be required to determine whether the mechanism presented for MolBC-A is common for the transport of even smaller substrates.

Acknowledgments—We thank K. J. Tanaka and C. Orelle for comments on the manuscript.

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