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SHORT COMMUNICATION

Dissection of the conformational cycle of the multidrug/lipidA ABC exporter MsbA

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ABSTRACT

Recent crystal structures of the multidrug ATP-binding cassette (ABC) exporters Sav1866 from *Staphylococcus aureus*, MsbA from *Escherichia coli*, *Vibrio cholera*, and *Salmonella typhimurium*, and mouse ABCB1a suggest a common alternating access mechanism for export. However, the molecular framework underlying this mechanism is critically dependent on assumed conformational relationships between nonidentical crystal structures and therefore requires biochemical verification. The structures of homodimeric MsbA reveal a pair of glutamate residues (E208 and E208′) in the intracellular domains of its two half-transporters, close to the nucleotide-binding domains (NBDs), which are in close proximity of each other in the outward-facing state but not in the inward-facing state. Using intermolecular cysteine crosslinking between E208C and E208C′ in *E. coli* MsbA, we demonstrate that the NBDs dissociate in nucleotide-free conditions and come close on ATP binding and ADP·vanadate trapping. Interestingly, ADP alone separates the half-transporters like a nucleotide-free state, presumably for the following catalytic cycle. Our data fill persistent gaps in current studies on the conformational dynamics of a variety of ABC exporters. Based on a single biochemical method, the findings describe a conformational cycle for a single ABC exporter at major checkpoints of the ATPase reaction under experimental conditions, where the exporter is transport active.

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Key words: ABC exporter; conformational dynamics; catalytic cycle; intermolecular cysteine crosslinking; multidrug transport.

INTRODUCTION

MsbA is an essential homodimeric ATP-binding cassette (ABC) transporter in *Escherichia coli* and other gram-negative bacteria, which mediates the transport of the Lipid A core moiety of LPS from the cytoplasmic membrane to the outer membrane.[1] It has been shown to also transport many substrates of the human multidrug resistance P-glycoprotein ABCB1,[2–4] and to exhibit an overlapping drug specificity with the bacterial homologues LmrA and Sav1866 from gram-positive *Lactococcus lactis* and *Staphylococcus aureus*, respectively.[4, 5] While much has been published regarding ATP hydrolysis by MsbA in the past few years,[2, 3, 6–9] recent focus has shifted to using this protein as a prototype ABC exporter to study the conformational movements associated with the catalytic cycle. This has primarily been done using X-ray crystallization,[10] cryo-electron microscopy (EM),[11] and spectroscopic techniques based on electron paramagnetic resonance (EPR) and double electron–electron resonance (DEER)[12–15] (Table I).

The first medium-resolution ABC exporter structure reported was the ADP-bound outward-facing state for Sav1866 in which the internal chamber between the membrane domains (MDs) faced the periplasm/exterior of the cell, and in which the nucleotide-binding domains (NBDs) were tightly dimerized.[17] However, the physiological relevance of this state was controversial due to contradictory biochemical studies indicating that the absence of the γ-Pi group destabilizes the NBD dimer.[18] As a result, Sav1866 was recrystallized with 5′-adenylyl-β-γ-imidodiphosphate (AMP-PNP) and because this conformation was also overall outward-facing, the ADP-bound structure was reinterpreted as the ATP-bound state[19] (Table I). The

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Table I
Published, Nucleotide-Dependent Conformations of Bacterial MsbA and Sav1866, and Mouse ABCB1a[a]

Experimental system, protein(s)	Protein conformation[b]			
	Nucleotide free	AMP-PNP[c]	ADP·Vi	ADP
A. X-Ray crystallization,				
i. Sav1866	n.a.[d]	Outward	n.a.	Outward
ii. MsbA	Inward	Outward	Outward	n.a.
iii. Mouse ABCB1a	Inward	n.a.	n.a.	n.a.
B. EPR/DEER,[e] MsbA in proteoliposomes	Inward	n.a.	Outward	n.a.
C. Cryo-electron microscopy, MsbA in proteoliposomes	n.a.	Outward	Outward	n.a.

[a]Table based on Ref. 16; see main text for references.
[b]Conformations are termed "outward" or "inward" depending on the side of the membrane (periplasm or cytoplasm, respectively) to which the internal chamber in the MDs is exposed.
[c]AMP-PNP, 5′-adenylyl-β-γ-imidodiphosphate; Vi, vanadate.
[d]n.a., not available.
[e]EPR, electron paramagnetic resonance; DEER, double electron–electron resonance.

authors further suggested that the outward-facing conformation with ADP was probably a result of the experimental drawback of having the protein in detergent solution.[19] Three MsbA structures were subsequently reported in distinct conformations, a nucleotide-free inward-facing (V-shaped, open-apo) state with the NBDs widely separated and predicted drug-binding sites in the MDs[2] facing the cytoplasm/interior of the cell for *E. coli* MsbA [Fig. 1(A)], and AMP-PNP-bound and ADP·vanadate (Vi)-trapped outward-facing states for *Salmonella typhimurium* MsbA[10] [Fig. 1(B)]. The latter two were identical to those obtained for Sav1866,[17, 19] but the study did not report an ADP-bound structure for MsbA. Recently, mouse multidrug resistance P-glycoprotein ABCB1a was crystallized in a nucleotide-free inward-facing state (Table I).[21] It is evident from Table I that no single ABC exporter has yet been crystallized at all stages of the ATPase reaction to generate a complete catalytic cycle.

Appreciating the importance of a physiological membrane environment for the conformational movements of transporters, EPR and DEER analyses on *E. coli* MsbA in proteoliposomes revealed that the separation distances between the NBDs in the nucleotide-free state and ADP·Vi-trapped state were in accordance with the crystal structures.[12–15] However, interdomain distance measurements of AMP-PNP- or ADP-bound forms of MsbA were not reported (Table I). Furthermore, cryo-EM was used on MsbA from *S. typhimurium* and *Vibrio cholera* reconstituted into proteoliposomes to study nucleotide-dependent conformations.[11] While the authors found minor distinctions between the AMP-PNP-bound and ADP·Vi-trapped structures in this work, they concluded that the overall conformations were similar to the crystal structures. In this case, nucleotide-free and ADP-bound conformations were missing (Table I). Thus, although major advancements have been made, till date no single technique has been used to investigate the conformations of a particular membrane-embedded ABC exporter at all four known checkpoints of the ATPase cycle (namely, nucleotide-free, ATP-bound, ADP·Pi-bound/hydrolysis intermediate, and ADP-bound) under physiological conditions suitable for transport.

In a recent molecular dynamics study on *S. typhimurium* MsbA, pairs of charged residues E208 and K212 in the cytoplasmic extensions of transmembrane helices 4/4′ of homodimeric MsbA, close to its NBDs, were implicated in electrostatic and hydrogen bond interactions at the central "tetrahelix bundle" in the outward-facing conformation of the transporter.[22] We hypothesized that cysteine residues at positions E208 and E208′ would be close enough to crosslink in this state (distance ~6 Å) [Fig. 1(B)] but not in the inward-facing conformation in which these residues are predicted to be separated widely (distance ~55 Å) [Fig. 1(A)]. Here, we have utilized E208C–E208′C disulfide crosslinking in homodimeric *E. coli* MsbA in a physiological phospholipid bilayer (plasma membrane vesicles) to detect the nucleotide-dependent conformations that are missing in current data sets.

MATERIALS AND METHODS

Bacterial strains and plasmids

L. lactis strain NZ9000 Δ*lmrA* Δ*lmrCD*,[23] which is devoid of the major endogenous multidrug transporters LmrA and LmrCD, was used as a host for pNZ8048-derived plasmids.[24] *E. coli* strain XL1 Blue was a host for the pGEM-5Zf(+) (Promega) cloning vector. Cells were grown in M17 medium (Oxoid) and LB medium (Formedium), respectively, as described previously.[2]

Site-directed mutagenesis

To create cysteine-less N-terminal His_6-tagged MsbA (MsbA-cl), endogenous C88 and C315 were each replaced by S in the wildtype (Wt) *E. coli msbA* gene in vector pGEMMsbA[2] (generating pGEMMsbA-cl) by site-directed mutagenesis using the QuickChange kit (Stratagene) with the forward primer 5′-CCA GCT ACT CTA TCT CCT GGG TAT CAG G-3′ and the reverse primer 5′-CCA GGA GAT AGA GTA GCT GGA GAC ATA G-3′ for C88S, and the forward primer 5′-GCG GCT TCT CAG ACG CTG TTT ACC ATT C-3′ and the reverse primer 5′-GCG TCT GAG AAG CCG CCA TAC CGC GCT G-3′ for C315S. The E208C mutant was generated by similar methods in pGEMMsbA-cl using the forward primer 5′-CCA CCA GCG CAT GCC AAA TGC TGA AG-3′ and reverse primer 5′-CTT CAG CAT TTG GCA TGC GCT GGT GG-3′. The *msbA-cl* and *msbA-cl E208C* genes were subcloned as NcoI-XbaI and NcoI-SacI fragments into pNZ8048 downstream of the nisin A-inducible promoter, yielding pNZMsbA-cl

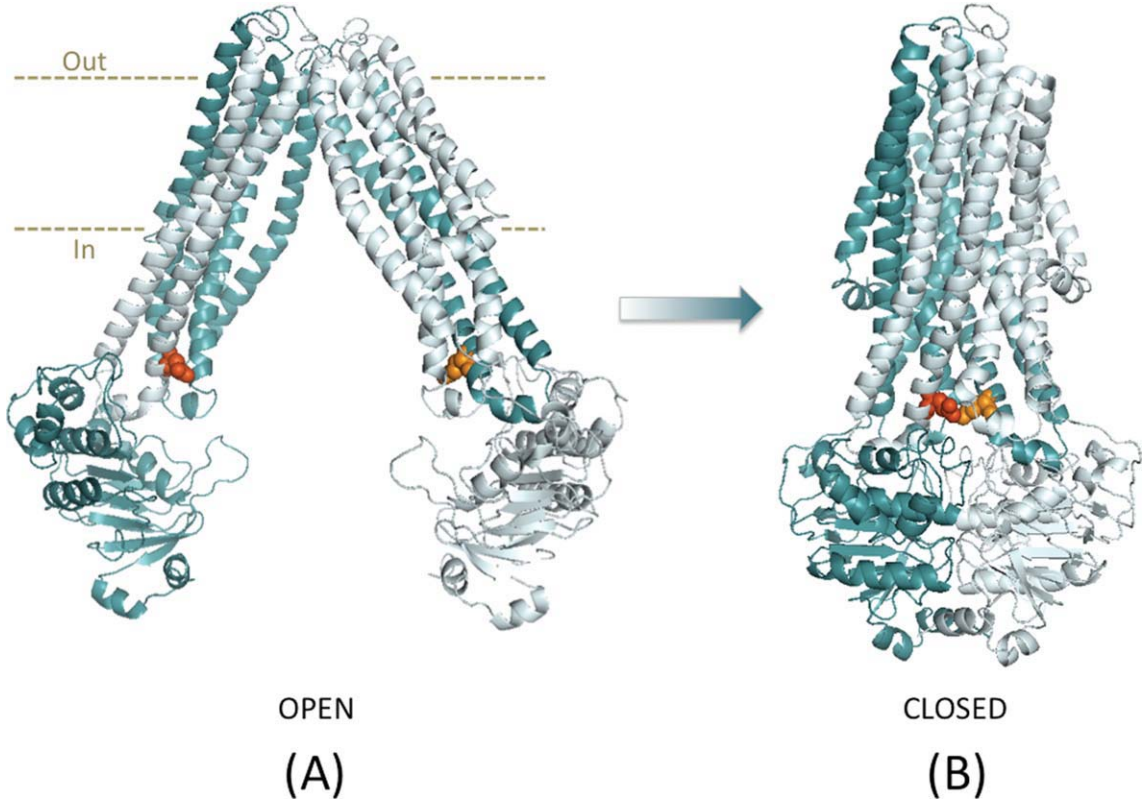

Figure 1

Positions of E208 and E208′ in homodimeric MsbA. **A**: The "open-apo" crystal structure of the *E. coli* MsbA dimer in a nucleotide-free inward-facing conformation (PDB 3B5W, full model) reveals residues E208 and E208′ (marked in red and orange) separated by more than 55 Å.[10] In and Out refer to the inside and outside of the plasma membrane, respectively. **B**: In the closed outward-facing AMP-PNP-bound crystal structure of *S. typhimurium* MsbA (PDB 3B60) in which the NBDs dimerize,[10] this distance is only about 6 Å. Figure was generated in PyMol.[20] Conformations are termed open (or inward-facing) and closed (or outward-facing) as described in the legend to Table I.

and pNZMsbA-cl E208C. The mutated *msbA* genes were sequenced to ensure that only the intended mutations were introduced.

Inside-out membrane vesicles (ISOVs)

MsbA proteins were expressed in the lactococcal cells by previously described methods.[2] ISOVs were prepared in 100 m*M* K-HEPES buffer pH 7.0 containing 5 m*M* $MgSO_4$ as previously reported.[2]

Transport assays

Hoechst 33342 transport assays in ISOVs in the presence of 2 m*M* nucleotides and ethidium transport assays in intact cells were conducted as described.[2]

ATPase activity measurements

ATPase activity in MsbA-containing ISOVs was measured using the malachite green colorimetric method.[23] Briefly, for each data point, 1 μg protein was added to 100 m*M* K-HEPES buffer pH 7.0, with 5 m*M* $MgSO_4$ and 2.5 m*M* ATP (high-grade ATP from Sigma). The assay was incubated for 5 min at 30°C, after which A_{600} was determined following the addition of malachite green-ammonium molybdate. The ATPase activity in ISOVs from non-MsbA–expressing cells was subtracted from the activity in MsbA-containing ISOVs and was typically about 40% of the latter.

Cysteine crosslinking

For each crosslinking reaction, ISOVs were diluted to 5 μg protein/μL in 100 m*M* K-HEPES buffer pH 7.0 containing 5 m*M* $MgSO_4$ in a reaction volume of 30 μL in microcentrifuge tubes with pierced lids. To reduce background signals due to crosslinks formed before nucleotide addition, 0.5 m*M* dithiothreitol (DTT) was added, and the samples were incubated at room temperature (RT) for 2 min. Nucleotides (2 m*M* AMP-PNP or ADP, or 2 m*M* ATP plus 2 m*M* sodium orthovanadate) were then added, and the crosslinking reactions were initiated by the addition of 0.5 m*M* copper phenanthroline solution, which was added from a 50 m*M* stock made by mixing $CuSO_4$ and 1,10 phenanthroline in a ratio 1:4 (w:w) in ultrapure H_2O.

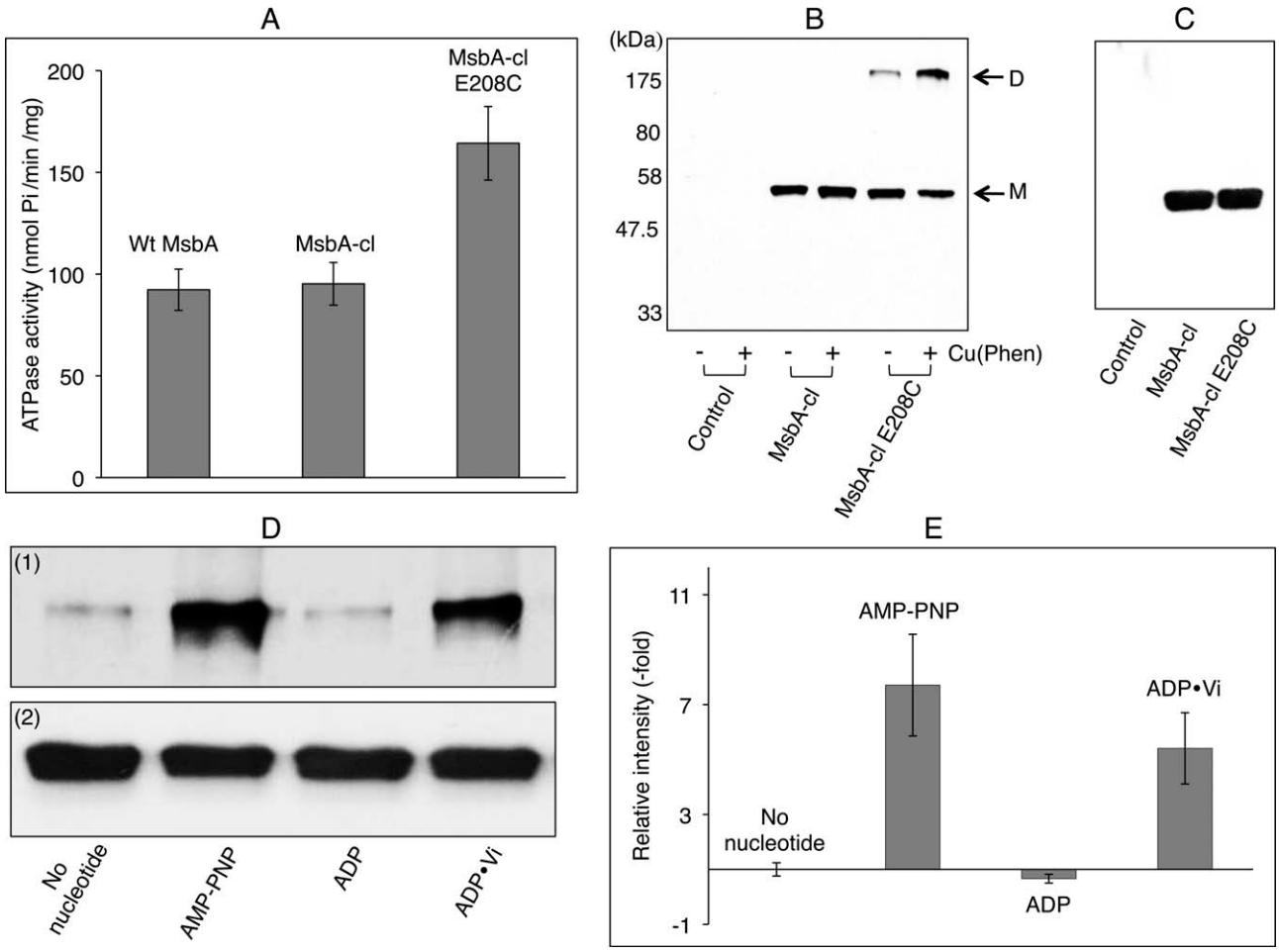

Figure 2

Crosslinking between E208C and E208C′ in homodimeric *E. coli* MsbA-cl. **A**: Background-subtracted basal ATPase activities of Wt MsbA, MsbA-cl, and MsbA-cl E208C in ISOVs. **B**: ISOVs without DTT contained self-crosslinked homodimeric MsbA-cl E208C. Crosslinking was enhanced by the addition of an oxidizing agent, copper phenanthroline (CuPhen). Subsequent to separation of total membrane proteins on SDS-PAGE, His_6-tagged MsbA proteins were detected on Western blot using an anti-pentaHis antibody. Control, ISOVs without MsbA. M and D refer to monomeric and homodimeric MsbA, respectively. Approximate positions of the molecular weight marker bands are shown alongside. **C**: No dimer bands were visible when ISOVs were mixed with 5× SDS loading buffer with DTT. **D**: (**1**) MsbA-cl E208C and MsbA-cl E208C′ crosslinked into dimers in a nucleotide-responsive manner. Background crosslinking was reduced with DTT prior to the inclusion of nucleotides. The reaction was started with Cu(Phen) and stopped with NEM. (**2**) Monomer bands of the same protein samples as in (1), after incubation with DTT but prior to crosslinking with CuPhen show equal loading into each lane. **E**: Densitometric analyses revealed clear distinctions between the proportions of MsbA dimers obtained under (D), presented as the relative signal intensity compared with the "no-nucleotide" signal (with a relative intensity of 1). Error bars represent ± SEM and are based on three independent experiments.

Some minor precipitates during the crosslink reactions were redissolved by mixing gently, and the reaction was allowed to occur for 5–7 min in a 30°C shaker incubator. The reactions were stopped by the addition of excess (10 m*M*) of the thiol alkylator *N*-ethylmaleimide and incubation at RT for 2 min. To avoid background crosslinking of denatured proteins and prevent breaking of formed disulphide crosslinks, 15 µg ISOVs from the crosslinking reactions were mixed with 5× SDS sample-loading buffer devoid of DTT and separated on an SDS-PAGE without any incubation. Gels were analyzed by Western blotting using horseradish peroxidase-conjugated anti-pentaHis secondary antibody and enhanced chemiluminescence (Kibbutz Beit Haemek, Israel). Band intensities on Western blot were compared by densitometric analyses using ImageJ software version 1.43 (NIH).

RESULTS AND DISCUSSION

Functional properties of MsbA-cl E208C

The E208C mutant was generated in MsbA-cl. MsbA-cl E208C was found to express at a similar level as MsbA-cl and Wt *E. coli* MsbA in the plasma membrane of *L. lactis*, and to mediate ethidium transport in intact cells and Hoechst 33342 transport in ISOVs (data not shown).[2,3] The mutant also exhibited ATPase activity in ISOVs [Fig. 2(A)]. Taken

together, these observations demonstrate that the MsbA-cl E208C mutant can be used to study catalytic cycle–dependent conformational changes in MsbA.

MsbA-cl E208C undergoes self-crosslinking

When total membrane proteins obtained from ISOVs from control cells without MsbA or cells expressing MsbA-cl or MsbA-cl E208C were separated on an SDS-PAGE gel (without DTT in the loading buffer) and subsequently analyzed by Western blotting, specific signals for the MsbA-cl and MsbA-cl E208C monomers were obtained in addition to a single band for the MsbA-cl E208C dimer [Fig. 2(B)]. The evidence that the dimer was due to intermonomer cys–cys crosslinking came from the observation that the dimer signal increased by exposure to oxidizing agent (copper phenanthroline) whereas the signal of the monomer decreased in the same sample [Fig. 2(B)]. Furthermore, the dimer band for the E208C mutant was not observed when ISOVs were diluted in sample-loading buffer containing DTT, prior to separation on an SDS-PAGE gel [Fig. 2(C)]. We made use of this observation to "reset" crosslinked MsbA dimers that were formed during the preparation of membrane vesicles, back to uncrosslinked dimers prior to examining nucleotide dependence of the crosslinking reaction.

Formation of intermolecular E208C crosslinks in dimeric MsbA-cl is responsive to nucleotides

Intermonomer crosslinking of MsbA-cl E208C monomers was tested in the presence of various nucleotides that represent intermediate steps of the ATPase cycle, namely AMP-PNP (for ATP-bound, prehydrolysis), ADP·Vi trapping (mimics the ADP·Pi transition intermediate) and ADP (posthydrolysis and Pi release). To initiate crosslinking after nucleotide addition, copper phenanthroline was added at a concentration equal to that of the DTT. In comparison with nucleotide-free conditions [Fig. 2(D)], we found a strong dimeric signal with the nonhydrolyzable ATP analog AMP-PNP. This result [Fig. 2(D)] and additional densitometric analyses of similar signals in independent experiments [Fig. 2(E)] indicated that a substantial proportion of MsbA dimers were trapped in the outward-facing conformation, allowing the engineered cysteines to come close enough for the formation of crosslinks [Fig. 2(D)]. A similar result was obtained with ADP·Vi trapping suggesting that MsbA remained in a similar overall conformation as observed with ATP binding [Fig. 2(D)]. However, we obtained a major reduction in the dimer signal when ADP alone was included in the crosslinking reaction [Fig. 2(D)]. Densitometry of signals in independent experiments indicated that the signal intensity was below that obtained for the no-nucleotide control [Fig. 2(E)], suggesting that ADP binding favors an inward-facing open conformation. Although the use of DTT and copper phenanthroline enhanced signal strength of crosslinking compared with background, a similar nucleotide dependence of crosslinking was observed in their absence.

Our crosslinking data address three important issues. First, the crosslinking data in the presence of AMP-PNP are in accord with the AMP-PNP-bound outward-facing crystal structure of Sav1866[19] and related structures for MsbA.[10] They support the idea that the outward-facing crystal structure of Sav1866 in the presence of ADP reflects either an ATP-bound or an ADP·Pi-bound state.[17] On the other hand, it might be possible that Sav1866 responds to the nucleotides differently than MsbA. Second, our signals in the presence of ADP support biochemical and structural evidence that the NBD dimer is not formed with ADP alone.[18] Third, our crosslinking data in the presence of ATP and Vi are in agreement with the notion that MsbA remains in an outward-facing conformation during the transition from the ATP-bound state to the catalytic transition (ADP·Pi bound) intermediate, consistent with observations on MsbA by cryo-EM[11] and by EPR and DEER spectroscopy.[12–15] Our findings point to a sustained dimerization of NBDs during ATP binding and ADP·Vi trapping, which complements EM analyses on human ABCB1[25] and MsbA.[11]

CONCLUSIONS

Ligand-driven conformational movements in ABC transporters are difficult to predict and study, and static crystal structures that hypothesize these conformational movements do require physiological verification. Using one and the same disulfide crosslinking technique, we were able to study MsbA dimerization at the major checkpoints of the ATPase reaction in plasma membrane vesicles and under conditions in which the transporter is transport active. This work provides a useful link between fragmented observations on nucleotide-dependent conformational movements in ABC exporters based on different techniques (X-ray crystallography, EPR/DEER spectroscopy, and EM).

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