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Minireview

The structures of MsbA: Insight into ABC transporter-mediated multidrug efflux

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Abstract ATP-binding cassette (ABC) transporters are integral membrane proteins that couple ATP hydrolysis to the transport of various molecules across cellular membranes. Found in both prokaryotes and eukaryotes, a sub-group of these transporters are involved in the efflux of hydrophobic drugs and lipids, causing anti-microbial and chemotherapeutic multidrug resistance. In this review, we examine recent structural and functional analysis of the ABC transporter MsbA and implications on the mechanism of multidrug efflux.

Keywords: Multidrug resistance; ABC transporter; Membrane protein; X-ray crystallography; MsbA; P-glycoprotein; LmrA

1. Introduction

Clinical multidrug resistance (MDR) represents a critical challenge in the treatment of human cancers and bacterial infections [1–5]. Drug-resistant bacterial strains that cause gonorrhea, pneumonia, cholera, and tuberculosis are widespread and difficult to treat. In humans, a similar mechanism of MDR is a major reason for the failure of numerous chemotherapeutics in the treatment of cancers. A major cause of bacterial and cancer drug resistance is the over-expression of a battery of MDR transporters that extrude toxic drug compounds out of the cell.

MDR transporters can be divided into two classes based on their source of energy: ATP-binding cassette (ABC) transporters which couple the hydrolysis of ATP to substrate transport across the cell membrane, and secondary transporters, which use proton gradients to facilitate transport. Over the past number of years, X-ray crystallographic studies of ABC transporters have added tremendous insight into understanding the underlying mechanics of ATP-mediated substrate transport to the already large body of genetic, biochemical and biophysical work. At the same time, many new questions have emerged that remain to be answered. Here, we review the three X-ray crystal structures of MsbA in the context of other structural and functional work.

2. ABC MDR transporters

ABC transporters compose one of the largest superfamilies of proteins [6,7]. Bacteria expresses two classes of transporters: importers and exporters, which are responsible for transporting a wide variety of substrates such as lipids, sugars, amino acids, peptides, ions and hydrophobic drugs. Eukaryotic genomes, on the other hand, only encode exporters (for a review of bacterial ABC transporters, see Davidson [8]; for a review of human ABC transporters, see Dean [9]). Exporters are responsible for conferring MDR phenotypes. ABC transporters are composed minimally of four core structural domains: two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs). ABC transporters in bacteria either express each of the four domains as separate subunit polypeptides, or a dimer with the NBD fused to the TMD. Eukaryotic ABC transporters are expressed either as dimers or as a single polypeptide containing all four domains. Structural divergence within the various subgroups of ABC transporters is prominent in the TMDs and likely reflects the large diversity of substrate specificity. The NBD, which is the hallmark of this transporter family, is more highly conserved. Functional MDR, has been identified in lipid flippases such as the human transporters P-gp (P-glycoprotein/MDR1/ABCB1) and ABCG2 as well as bacterial transporters LmrA and MsbA, both in vitro and in vivo [10–17] (Table 1).

3. MsbA, a lipopolysaccharide flippase/MDR homologue

MsbA, an essential ABC transporter in Gram-negative bacteria, is a lipid flippase that transports lipid A and lipopolysaccharide (LPS), the major component in the bacterial outer membrane, from the inner to the outer leaflet of the inner membrane [18,19]. In vitro studies have demonstrated that MsbA is an ATPase that is specifically stimulated by lipid A and LPS molecules [20] and has overlapping substrate specificities with both LmrA and P-gp [16]. A recent paper by Woebking et al. has shown that MsbA interacts with multiple drugs, and confers an increase in the level of resistance to the antibiotic erythromycin when expressed in Gram-positive *Lactococcus lactis* [17]. Thus, MsbA expressed in an LPS-free environment is a

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Abbreviations: ABC, ATP binding cassette; ICD, intercellular domain; LPS, lipopolysaccharide; MDR, multidrug resistance; NBD, nucleotide binding domain; P-gp, P-glycoprotein; TMD, transmembrane binding domain; EM, electron microscopy

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Table 1

Transporter	MDR substrates	Known non-MDR substrates	Reference
P-gp (MDR1/ABCB1)	Duanorubicin, doxorubicin, vinblastine, vincristine, actinomycin-D, bisantrene, docetaxel, etoposide, homoharringtone, paclitaxel, teniposide	Neutral and cationic organic molecules, phospholipids Hoechst	[58]
MsbA	Duanorubicin, doxorubicin, vinblastine, erythromycin, TPP^+, ethidium bromide	Lipid A, LPS Hoechst	[16,17]
LmrA	Duanorubicin, doxorubicin, vinblastine, TPP^+, erythromycin, tetracycline, tetramethylrosamine, benzalkonium, ethidium bromide	Lipids Hoechst	[14,59–61]

competent multidrug transporter. This suggests that the underlying transport mechanism between MsbA, LmrA and P-gp is likely conserved, with an evolutionary fine tuning in the specificity for the native substrates such as LPS, in the case of MsbA. In addition, MsbA from *E. coli* shares one of the highest protein sequence similarities to P-gp (36% identical to the NH_2-terminal half of human P-gp) further indicating a common evolutionary origin for the mechanics of amphipathic and hydrophobic substrate transport.

4. Driving translocation with ATP hydrolysis

The NBD, which is also called an ABC, is the unifying feature of the ABC transporter family. The high resolution X-ray crystal structures of isolated NBDs (HisP [21], MalK [22,23], MJ1276 [24], MJ0796 [25,26], TAP1 [27] GlcV [28], HlyB [29,30] and CFTR [31,32], to name a few) have shown three conserved structural subdomains: an F1-type ATPase binding core containing the Walker A (P-loop) and Walker B ATPase motifs, an antiparallel β-sheet subdomain that positions the base and ribose moieties of the nucleotide, and an α-helical subdomain that contains the ABC signature or 'LSGGQ' motif distinct to the ABC transporter family (Fig. 1).

A picture of the various conformations along the ATP hydrolysis cycle has begun to emerge from the combination of structures from isolated NBDs and intact ABC transporters. The structure of the full-length bacterial vitamin B12 ABC importer, BtuCD, shows two interacting NBDs forming a dimer in the absence of nucleotide [33] (Fig. 1A). The isolated NBD structure of ATP-bound MJ0796, reveals an active site architecture with the P-loop and the 'LSGGQ' signature motif anchoring and aligning the bound ATP molecules to form two composite active sites [26] (Fig. 1B). Finally, the NBDs from the ADP, orthovanadate structure of intact ABC transporter MsbA, form a dimer interface similar to both BtuCD and MJ0796, however the 'LSGGQ' motif does not interact with the nucleotide and vanadate (Fig. 1C) [34]. In the MsbA ADP-vanadate structure, the presence of only one bound nucleotide per NBD dimer is observed and consistent with numerous studies of ABC transporters such as P-gp [35] and MalK [36,37] in which vanadate enters the active site of only one NBD to form a complex with ADP. Although all of the solved X-ray structures of intact ABC transporters (BtuCD and MsbA) were solved at medium resolution and therefore a degree of uncertainty may be present in the side chain positions, the value of these structures is to provide insight into gross conformational changes associated with the ATP hydrolysis cycle in the NBDs.

Based on the available structural data, we propose that opening and closing of the NBD dimer interface drives conformational changes in the TMDs and therefore translocation of substrate. The conformation of NBDs in the BtuCD structure may represent the initial state of dimerization in which the NBDs are in close contact. ATP binding likely decreases the distance between the interacting NBDs and drives conformational changes in the TMDs. This tight dimerization as observed in the MJ0796 structure could be broken during hydrolysis by buildup of negative charge on the leaving γ-phosphate that causes a disengagement of the interacting motifs. This would result in the conformation observed for MsbA with ADP and vanadate. Asymmetric hydrolysis of ATP supported by the MsbA structure, would couple an 'alternating' catalytic cycle to the transport of drugs [38]. One possibility is that asymmetric substrate binding to the TMDs would force the deformation from two kinetically indistinguishable NBDs

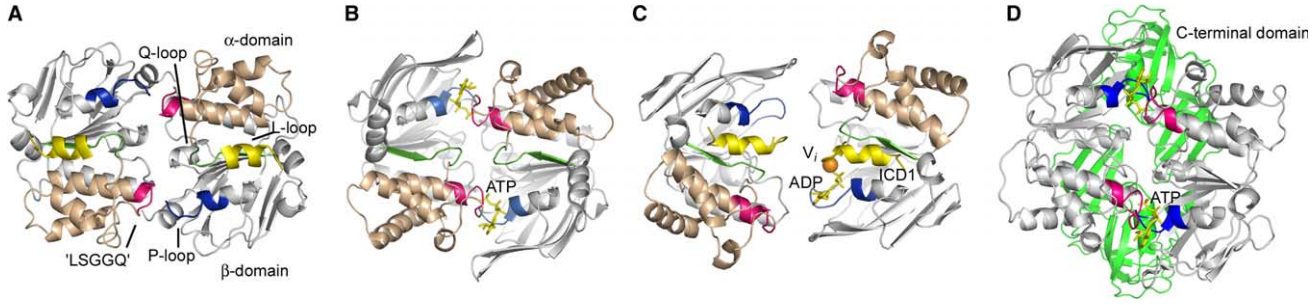

Fig. 1. Structures of the NBDs of ABC transporters. (A) BtuCD (1L7V). The α-domain and β-domains are colored in tan and gray, respectively. P-loop or Walker A (blue), Q-loop (green), 'LSGGQ' motif (magenta), ATP (yellow), and L-loop (yellow) are shown. (B) ATP-bound homodimer from MJ0796 (1L2T). (C) MsbA in complex with ADP and orthovanadate (orange sphere) (1Z2R). The ICD1 helix is colored yellow. (D) MalK (1Q12). The C-terminal domain is highlighted in green. All pdb codes are in parenthesis. The image was created using the program Pymol.

to conformationally non-equivalent ATP binding sites, thus breaking the symmetrical conformation of the NBDs. The breaking of the symmetry in the NBDs would then in turn drive large conformational changes in the TMDs. A biochemical study of the stochiometry between ATP and substrate in OpuA, an osmoregulated ABC transporter, reveals that two ATPs are required for the transport of one substrate. This is consistent with the idea that the first ATP is hydrolyzed during transport and second ATP as a reset mechanism, restoring symmetry to the nucleotide-binding sites [39,40]. An alternative to this model has been proposed whereby the binding of nucleotide rather than ATP hydrolysis initiates drug transport [41–43].

A critical issue to the ABC transporter field is the mechanism of activation of ATP hydrolysis and the role that substrate binding plays in this. Recent structural and biochemical studies of specific mutations in the ATPase catalytic site have begun to clarify the mechanism of activation. Mutation of a conserved glutamate residue in the Walker B motif, designated the 'catalytic carboxylate,' hinders the formation of the closed conformation of the NBDs normally associated with ATP binding [44]. A double mutant of this glutamate promotes the formation of what is termed the 'occluded nucleotide conformation' in which an NBD dimer with tight ATP binding is formed [45]. Substrate binding further promotes the formation of this conformation and the authors suggest that after initial loose binding of ATP in the two NBDs, drug or substrate binding drives the formation of the occluded conformation, with one tightly bound ATP committed to hydrolysis. In the recent structure of the $ATP \cdot Mg^{2+}$ bound NBD dimer of HlyB, Zaitseva et al. propose that an active site histidine (H662 in HlyB, H537 in MsbA) coordinates with the 'catalytic carboxylate' glutamate to form a catalytic dyad critical for accurate positioning of the nucleotide [30]. The available structural and biochemical data strongly suggest that both nucleotide and substrate binding are required to position the active site moieties to ensure the electrostatic and/or structural integrity of the active site and contribute to the formation of an active NBD dimer. We propose a 'step-wise' activation of ABC transporters through discrete conformational stages during formation of the active NBD dimer. Thus, activation requires conformational changes across the NBD dimer and TMD/NBD interface that coordinates the positioning of catalytic residues in both NBD active sites. Each stage provides a potential control point in the translocation reaction at which regulation by the various components can be exerted, thus ensuring the binding and translocation of substrate at the appropriate time. Clearly, significant effort will be required to bridge the gap between the structures of isolated NBDs, which provide atomic resolution but are limited in the physiological interpretation, and the medium resolution structure of these intact transporters, which provide data on the domain architecture and conformational changes but lack the precision of side chain positioning.

5. Substrate specificity and translocation pathway

The role of the TMD is to recognize and mediate the passage of substrates across cell membranes. For ABC transporters that extrude hydrophobic or amphipathic molecules, the TMD shields the polar groups of the substrates from the lipids making up the bilayer, while accommodating the hydrophobic groups to provide a pathway through the cell membrane. The TMDs for drug and lipid flippases are capable of recognizing and removing a large number of chemically unrelated lipids and toxins directly from the cell membrane [46]. The X-ray structures of MsbA provide an initial framework to understand the dynamic nature of the conformational changes in the TMDs involved in mediating substrate transport.

The overall structural organization of MsbA was revealed in two different nucleotide free structures, one in an open conformation and the other closed (Fig. 2A and B) [47,48]. These X-ray structures reveal a chamber formed from twelve transmembrane α-helices. The 'open' conformation exhibits an inverted 'V' shape with an opening accessible to the interior of the transporter and dimer contacts concentrated between the extracellular loops. In the 'closed' conformation, the transporter forms extensive contacts within the dimer interface of the TMDs. In addition, the openings of the chamber are defined by intermolecular contacts between TM2 from one monomer and TM5 from another. The chamber is lined with charged and polar residues that are likely solvated, creating an energetically favorable environment for polar moieties in drug compounds and sugar groups from LPSs. The large size of this polar chamber could give a structural basis for multi-substrate specificity.

The recent X-ray structure of MsbA in complex with ADP, orthovanadate and Ra LPS reveals a dramatic conformational change in the TMDs that drives substrate transport. A large torque of the TMDs accompanies this post-hydrolysis transition state, closing the cytoplasmic accessible chamber and shifting the accessibility of this chamber to the extracellular side of the cell membrane (Fig. 2C). LPS is bound to the membrane-exposed sides of the protein at the dimer interface composed of TM1, TM5 and TM6 from one monomer and TM2 from the other monomer. This binding site corresponds to a 'hot-spot' of conserved residues shared amongst P-gp, LmrA and MsbA [34]. In addition, the conformational changes in the TMDs are consistent with conformational changes observed in P-gp after ATP hydrolysis [49].

6. Mediating communication between NBDs and TMDs

A central question for ABC transporters is the mechanism of transmitting substrate binding and ATP hydrolysis between the NBDs and the TMDs. Based on the MsbA and BtuCD structural work, we suggest that a conserved helix that extends from the TMD segments into or near the active site of the NBD is largely responsible for the inter-domain crosstalk. In MsbA, the intercellular domain helix 1 (ICD1) (residues 97–139), is in contact with the NBD and forms a U-like structure consisting of three α-helices. The first and third helices are extensions of TM2 and TM3; the second helix of ICD1 rests in the active site of the NBD and serves as a potential pivot about which the NBD could rotate. BtuCD also has an analogous structural motif mediating contact between the NBD and the TMD [33]. However, in both the absence of substrate and nucleotide, the ICD1-like motif in BtuCD is positioned out of the active site. In the nucleotide-bound structure of MsbA, the ICD1 contacts the Q-loop, which contains a conserved glutamine. In the MJ0796 ATP-bound dimer structure, this glutamine joins with a conserved glutamate from the Walker B motif to coordinate a water positioned for nucleophilic attack

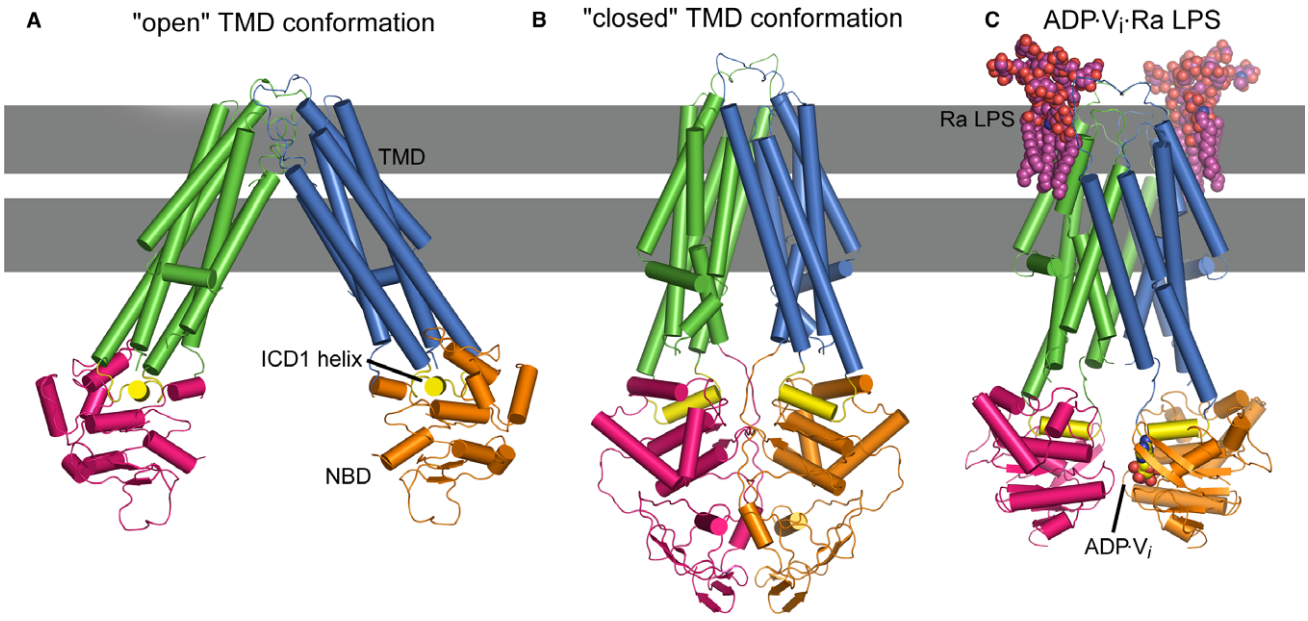

Fig. 2. X-ray crystal structures of MsbA. TMDs are colored green and blue, NBDs are colored magenta and orange. ICD1 helix is colored yellow. (A) "Open" conformation of apo MsbA from *E. coli* (1JSQ). (B) "Closed" conformation of apo MsbA and *V. cholera* (1PF4). (C) ADP · V_i-bound structure of MsbA from *S. typhimurium* with bound Ra LPS shown in magenta.

of the γ-phosphate. This suggests that the ICD1 helix could communicate the catalytic state of the nucleotide to the TMDs (Fig. 2B). Another interesting and more likely possibility is that the presence of substrate is communicated through the ICD1 helix to the NBDs which in turn promotes the correct positioning of catalytic residues in the various conserved motifs present in the NBDs.

7. Conformational dynamics of ABC transporters

Recent EPR studies of MsbA by Dong et al. [50], have used site-directed spin labeling to characterize the conformational changes in apo and nucleotide bound forms of MsbA. Accessibility to molecular oxygen and NiEDDA by 112 spin-labeled mutants on TM2, TM5 and TM6 suggest that MsbA is in a dynamic equilibrium of conformations that includes, but is not limited to, the two apo X-ray structures. They also observe a solvent filled chamber accessible to the cytoplasmic ends of the TMDs in the absence of nucleotide, which is also consistent with the apo structures. In addition, Buchaklian et al. showed that site-directed spin labeling and cross-linking results were consistent with the TMDs in the resting state conformation of the MsbA observed in the apo crystal structures [51]. Electron microscopy structures of other putative MDR-ABC transporters have also demonstrated that these proteins can undergo significant conformational changes. The EM structure of the bacterial ABC transporter YvcC from *Bacillus subtilis* has revealed an 'open' conformation that agrees well with the X-ray structure of the 'open' apo-state of MsbA and shows that the NBD can disengage from the ICD during the catalytic cycle [52]. In addition, the EM reconstruction of Pdr5p has also been determined [53] and is consistent with the 'open' apo structure of MsbA. Nevertheless, the physiological nature of the 'open' apo conformation of these transporters remains controversial. The TMDs could sample such a large conformational space in order to filter substrates from the lipid bilayer, but this might depend on the speed of turnover of the transporter.

The X-ray structure of MsbA in complex with ADP and vanadate shows that large conformational changes in the TMDs are dependent on the nucleotide bound state of the NBDs. In addition, the EM reconstruction structure of P-gp in complex with the non-hydrolyzable analogue, AMPPNP at 8 Å shows a domain organization similar to MsbA, and an overall arrangement between the two halves that is more consistent with the ADP · vanadate structure of MsbA than either of the two apo structures [54]. The EM structure of P-gp is also the first structure of an intact ABC transporter to show an asymmetric organization between the two TMD halves but a symmetric dimer of NBDs, raising the interesting question as to the mechanistic importance of this asymmetry in the absence of bound translocation substrate.

The NBDs are also dynamic in nature. Purification of isolated NBDs is highly problematic in the absence of nucleotide [55]. This empirical observation suggests, in combination with the unfolded nature of the NBDs in the apo MsbA structures, that the NBDs are intrinsically unstable in the apo form. The concentration of nucleotide in the cell is sufficiently high to conclude that the NBDs would not sample such a large unfolding between catalytic cycles as observed in the apo structures of MsbA. However, disengagement of the NBDs from the TMDs and a relaxation of the α and β subdomain packing in the absence of nucleotide and/or substrate is possible. This could provide a potential regulatory point during substrate transport and would require discreet conformational changes during drug/substrate binding, ATP binding, ATP hydrolysis and nucleotide release.

One outstanding issue is the conformational changes associated with a catalytic transition state. Dong et al.'s EPR studies on the ATP-vanadate state shows a shift in accessibility of the extracellular domains that is consistent with the post-hydroly-

sis transition state structure of MsbA. However, the dimer organization of the NBDs in the ADP-vanadate structure of MsbA does not agree with a previous study by Fetsch and Davidson [56] of vanadate induced photo-cleavage of the bacterial importer NBD subunit MalK, in which vanadate-induced cleavage occurs in both the P-loop and 'LSGGQ' signature motif suggesting that the two motifs in MalK are in close proximity to the nucleotide and vanadate ion. In the current structure of MsbA, the P-loop and the signature motifs are disengaged and an anomalous peak for the vanadate ion is coordinated to the ADP but not in close proximity to the 'LSGGQ' signature motif of the opposite dimer.

We offer a possible explanation for the discrepancy between the MalK studies and the MsbA studies. Hydrolysis of ATP is a multi-step mechanism that results in discrete conformational changes in the NBDs. The inherent differences within the various subgroups of ABC transporters (in particular between importers, like MalK, and exporters, like MsbA), would amplify these conformational differences and result in vanadate entering more favorably into the γ-phosphate position at an earlier stage in the MalK case and in a latter stage in the MsbA case. In addition, in MalK a C-terminal domain increases the affinity between MalK-NBD subunits. Upon examination of the MalK structure, it is evident that this domain bridges the two interacting NBDs and imparts unique properties to this ABC importer (Fig. 1D). Although most isolated NBDs purify as monomers in the absence of nucleotide, the MalK purifies as a dimer [23]. Such a significant structural feature that stabilizes the dimeric form of the MalK-NBDs, even in the absence of nucleotide, would likely also play a role in stabilizing a closed dimer in the catalytic transition state. This C-terminal domain is not present in MsbA, P-gp or LmrA and therefore the observation of vanadate cleavage in the 'LSGGQ' motif of MalK may be specific to NBDs with stabilizing domains that act to hold the NBDs together. This may be an adaptation to ensure fidelity in the absence of a polypeptide tether between the various subunits in most ABC importers.

8. Model mechanism for substrate "flipping"

MDR-ABC transporters sequester lipid/drug molecules as they diffuse through the cell membrane and either flip them to the outer leaflet or transport them into the extracellular milieu. In order to accomplish this, they must first recognize a diversity of substrates, and then partially sequester the polar groups from the membrane in a manner that would allow an energetically favorable movement to the outer leaflet of the cellular membrane. In our previous work, we presented a putative mechanism where upon binding substrate, MsbA adopts a closed conformation that traps the substrate within the chamber and transports it exclusively through this chamber. However, the structure of MsbA in complex with LPS and nucleotide suggests an alternative mechanism of substrate "flipping".

We propose a model of substrate "flipping" where the sugar head groups of the LPS molecules are sequestered and then diffuse across the membrane in the internal chamber of MsbA while the hydrophobic tails of the lipid are dragged through the bilayer. We have extended this mechanism to encompass drug compounds involved with MDR, which also have the property of being amphipathic. Based on a locus of conserved residues between MsbA, LmrA and P-gp and residues that have been identified in drug binding studies for P-gp (for a detailed examination please see, [49,57]), we propose that substrate initially binds near the elbow helix. During the "power stroke", the polar groups are sequestered within the chamber and moved to the outer leaflet of the membrane by the rigid body shearing of the TMDs while the hydrophobic groups are dragged through the lipid bilayer. There also may be more than one pathway through the dimer. The internal reorientation of the TM5 and TM6 upon ATP hydrolysis may act as a gate allowing the substrate to move from the inner leaflet to the outer leaflet, through channels on either side of the central pore.

9. Concluding remarks

The recent X-ray structures of MsbA together with biophysical and biochemical studies provide insight into the conformational flexibility and mechanistic properties of MDR ABC transporters. However, many critical issues still remain: (i) What are the specific molecular components that direct signal through the ICD1 helix to the NBD active site? Is this substrate-driven or nucleotide-driven or both? Is this conserved in the entire ABC transporter family? (ii) What are the discrete conformational changes in the NBD dimer that are associated with the various stages of ATP hydrolysis? If substrate binding initiates hydrolysis of ATP in an asymmetric manner, and two ATP molecules are hydrolyzed per single substrate transported, how is hydrolysis initiated in the second NBD? (iii) What is the conformation of the TMDs in the ATP dimer state? Is this the transport "power stroke"? Structural and biochemical studies must be directed at these and other key questions to further drive our understanding for this class of clinically important integral membrane proteins.

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