The Conformational Transition Pathway of ATP Binding Cassette Transporter MsbA Revealed by Atomic Simulations

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ATP binding cassette transporters are integral membrane proteins that use the energy released from ATP hydrolysis at the two nucleotide binding domains (NBDs) to translocate a wide variety of substrates through a channel at the two transmembrane domains (TMDs) across the cell membranes. MsbA from Gram-negative bacteria is a lipid and multidrug resistance ATP binding cassette exporter that can undergo large scale conformational changes between the outward-facing and the inward-facing conformations revealed by crystal structures in different states. Here, we use targeted molecular dynamics simulation methods to explore the atomic details of the conformational transition from the outward-facing to the inward-facing states of MsbA. The molecular dynamics trajectories revealed a clear spatiotemporal order of the conformational movements. The disruption of the nucleotide binding sites at the NBD dimer interface is the very first event that initiates the following conformational changes, verifying the assumption that the conformational conversion is triggered by ATP hydrolysis. The conserved x-loops of the NBDs were identified to participate in the interaction network that stabilizes the cytoplasmic tetrahelix bundle of the TMDs and play an important role in mediating the cross-talk between the NBD and TMD. The movement of the NBD dimer is transmitted through x-loops to break the tetrahelix bundle, inducing the packing rearrangements of the transmembrane helices at the cytoplasmic side and the periplasmic side sequentially. The packing rearrangement within each periplasmic wing of TMD that results in exposure of the substrate binding sites occurred at the end stage of the trajectory, preventing the wrong timing of the binding site accessibility.

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uted to a typical outward-facing conformation. The NBD dimer adopts a canonical head-to-tail closed form with two nucleotide molecules binding at the interface. The transmembrane helices split into two groups forming two wings at the periplasmic side, resulting in the opening of the putative translocation pore to the periplasm (Fig. 1a). At the cytoplasmic side, TM3 and TM4 helices from two TMDs pack tightly, forming a tetrahelix bundle, which occludes the cytoplasmic gate of the central pore. Moreover, the TMD dimer adopts a domain-swapping arrangement with TM4/TM5/IH2 crossing over to associate with the opposing monomer, a structural character first observed in Sav1866 (14). The nucleotide-free closed-apo structure of MsbA has an inward-facing conformation (Fig. 1b). The central pore closes at the periplasmic side and opens to the cytoplasmic side. Compared with the nucleotide-bound outward-facing conformation, the packing of transmembrane helices of this inward-facing structure has been rearranged. The spacing between TM1 and TM6 at the periplasmic side disappears, and a new gap emerges between TM4 and TM6 at the cytoplasmic side. The NBD subunits are misaligned with two P-loops in close proximity and the ATP binding sites disrupted. With this structural information, the “alternating access” model (35) with conformational exchange between the outward-facing and inward-facing states is generally supported in MsbA as in other ABC transporters. In this model, substrate binds to the inward-facing MsbA and is exported to the periplasmic side through the conversion of the TMDs to the outward-facing state. The transporter then uses the energy of ATP hydrolysis to return to the initial inward-facing state, finishing a cycle of transport. However, the atomistic picture of the conformational conversion remains elusive, and it is especially intriguing for MsbA as it serves as a prototype of ABC exporter and is homologous to human MDR1.

Because conventional molecular dynamics simulation in explicit water solutions can access only a limited time scale (~100 ns), simulating such large-scale conformational movement as in the case of MsbA is generally not feasible (36–38). Here we resort to the targeted molecular dynamics method, which is effective in generating a trajectory from a known initial structure to a known target structure and getting insights into the atomistic details of the conformational transition (39–43). In this report a targeted molecular dynamics study of the conformational transformation of MsbA from the outward-facing state to the inward-facing state was presented. This represents the process that resets the transporter to the initial substrate-accessible state by using the energy of ATP hydrolysis. The simulation trajectories revealed that the conformational transition has a clear spatiotemporal order in which the timing of the conformational movement steps depicts an allosteric signal pathway across the protein from NBDs to TMDs. In the dynamic picture produced by the simulations, the unique structural arrangement of the MsbA type exporter and its intrinsic con-

**FIGURE 1.** Simulation system of outward-facing (initial) (a) and inward-facing (b) conformations of MsbA exporter buried in the bilayer membrane. For clarity, only a part of the water molecules and POPC lipids (with phosphorus atoms enlarged) in the system is depicted. TM3, TM4, Walker-A, and LSGGQ motifs are highlighted in black.
formational coupling relationship between each part of the protein manifest their functional importance.

**EXPERIMENTAL PROCEDURES**

All simulations were carried out by using NAMD 2.6 (44) and the CHARMM27 force field (45). Simulation conditions were maintained at 1.01325 bar by using the Nosé-Hoover Langevin piston method (46, 47) and at 300 K by Langevin dynamics with a damping coefficient of 1.0 ps⁻¹. For the non-bond interactions, the switching function began to take effect at 10 Å, and the cut-off was set to 12 Å. Electrostatic interactions were represented using the particle mesh Ewald method with a grid spacing of 1 Å. The data analyses were done by using VMD (48).

The nucleotide-bound crystal structure of MsbA was taken from PDB data base (PDB code 3B5X). The AMPPNP molecules were replaced by ATP molecules, and the Mg²⁺ ions were added to the nucleotide binding sites according to the position of the Na⁺ ion in the crystal structure of MJ0796 (PDB code 1L2T). All titratable residues were kept in their default state. The MsbA protein was buried into pre-equilibrated POPC bilayer following the “shrinking” method (49). The protein was first centered in the expanded bilayer (expanded in the xy plane by four times), and the lipids within 14 Å of protein were removed. Then the “compression” steps were performed with a scaling factor of 0.98 and a minimization step of 500. The structure after 67 cycles was selected, then it was solvated by TIP3 water (50) and neutralized by adding 4 chloride ions. The final system contains 131,872 atoms including 249 POPC lipid molecules in a rectangular box (~94 × 94 × 150 Å in size). The system was first minimized by 5000 steps with the heavy atoms of protein fixed. Then the solvent was relaxed for 200 ps, and the lipid molecules and solvent were further equilibrated for 2 ns. The restraining force on protein was reduced to 0 in 160 ps, and then the whole system was equilibrated for 4 ns without any constraint. The integration step was set to 2 fs, and the SHAKE algorithm (51) was used to constrain all hydrogen atom-containing bonds. The MsbA system appears stable in the 4-ns simulation as an NPT ensemble. The Cᵣ root-mean-square deviation (r.m.s.d.) value relative to the initial crystal structure of the integral MsbA fluctuates around 2.7 Å in the last 2 ns and is below 3 Å in most of the simulation times. Simulations were also performed in the nucleotide-free state by removing the ATP molecules and Mg²⁺ ions from the final structure of the 4-ns equilibration run, and the whole system was further equilibrated with the harmonic constrain forces on the protein. Constraints were gradually diminished to zero in 160 ps, and the final structure was used as the nucleotide-free initial structure for targeted MD.

Targeted MD propels a known initial structure to a known target structure by use of an external potential (52). The potential is made to decrease r.m.s.d. of the system relative to the target structure toward a preset value at each step. The potential used can be described as

$$U_{\text{MD}} = \frac{k}{2N} \left[ r_{\text{m.s.d.}}(t) - r_{\text{m.s.d.}*}(t) \right]^2$$  \hspace{1cm} (Eq. 1)

where \( r_{\text{m.s.d.}}(t) \) is the instantaneous best-fit r.m.s.d. of the current coordinates to the target coordinates, \( r_{\text{m.s.d.}*}(t) \) is the preset r.m.s.d. value for the current time step, and \( k \) is the force constant and \( N \) the number of targeted atoms.

Totally nine targeted MD trajectories were conducted, including four 500-ps and one 1-ns trajectories without nucleotide molecules, three 500-ps trajectories with ATP bound, and one 500-ps trajectory with ADP bound. The 500-ps trajectories with different initial velocities gave very similar results as well as the 1-ns trajectory, showing that the simulation time of 500 ps is sufficient. The existence of nucleotide molecules perturbed the NBD interface disruption step (see below); however, the general picture of the whole process was maintained. Details of all nine trajectories are summarized in supplemental Table S1. The following analyses and all the figures are based on one of the 500-ps trajectories without nucleotide molecules (trajectory I in supplemental Table S1).

According to the targeted MD trajectories, the conformational transition of MsbA from outward-facing to inward-facing state can be ordered as a series of events, the time scale of which is shown in Fig. 2. Disruption of nucleotide binding sites and the NBD dimer interface is the first remarkable event. After that, the interactions between TMD and NBD are weakened, and the tetrahelix bundle network at the cytoplasmic side of TM3 and TM4 breaks down followed by rearrangements of the cytoplasmic side of TM helices. The packing rearrangement of TM helices within each wing at the periplasmic side occurs last, although the shutting motion of the two wings starts at the very beginning and lasts throughout the simulation. In the following sections, we will describe these motions in detail.

**Disruption of NBD Dimer Interface**—The disruption of NBD dimer interface is the first significant step along the targeted MD trajectory. Some interaction pairs across the NBD dimer interface are common in ATP-bound, ADP-bound, and nucleotide-free systems, including hydrogen-bond interactions between the x-loops of each NBD (Asn-477ᴬ/ᴮ⁻Arg-377ᴬ/ᴮ, Asn-477ᴮ/ᴬ⁻Asp-553ᴮ/ᴬ, and Ser-482ᴮ⁻Ser-378ᴬ/ᴮ) and the salt-bridge interactions between α3 and β6 (Lys-465ᴮ⁻Asp-553ᴮ/ᴬ). The non-bond interactions between the Walker A and LSGQG motif (Ser-378ᴬ⁻Ser-378ᴮ⁻Ser-482ᴮ/ᴬ) and the salt-bridge interactions between α3 and β6 (Lys-465ᴮ⁻Asp-553ᴮ/ᴬ) and between the Walker A and D-loop (Arg-377ᴮ⁻Glu-514ᴮ/ᴬ). The x-loop preceding the LSGQG signature motif is highly conserved among ABC exporters (14), and the Asn-477ᴮ/ᴬ⁻Asn-477ᵀᴹ hydrogen bond just beneath the tetrahelix bundle...
connects the x-loops from each NBD subunit. In the absence of ATP, these interaction pairs contribute to stabilize the head-to-tail closed arrangement of the NBD dimer. At about 65 ps of the targeted MD simulation, the cross-interface interactions between the NBD subunits began to fall apart. The hydrogen bond Asn-477<sub>A/B</sub>-Asn-477<sub>B/A</sub> and the salt bridge Lys-465<sub>A/B</sub>-Asp-553<sub>B/A</sub> broke earlier, whereas the other interactions around nucleotide binding sites lasted longer, and one of them even held until 150 ps (Fig. 3c). The total disruption of these intersubunit interactions characterizes the dissociation of the nucleo-

FIGURE 2. The time table of the conformational changes of different parts of the transport system during the outward-facing to inward-facing transformation. The period of each event is represented by a straight line with the starting time and end time labeled.

FIGURE 3. The topview (a) and frontview (b) of NBD dimer interface are shown. Walker-A, LSGGQ motifs, and x-loop of chain A are highlighted in darker gray. The electrostatic and hydrogen-bond interaction pairs across the dimer interface are highlighted with frames. c, variation of some hydrogen bonds between NBD monomers, x-loop and IH2, or the TM helices of the tetrahelix bundle throughout the simulation time is shown. Each grid represents 5 frames (2.5 ps), and its gray level corresponds to the percentage of hydrogen bond emergence in this period (black, ≈ 60%; dark gray, 40%; gray, 20%; light gray, none). The hydrogen bond cutoffs are set by the donor-acceptor distance being less than 3.5 Å and the donor-hydrogen-acceptor angle being less than 30°. d, shown is the variation of the distance between mass center of Walker A motif and that of the LSGGQ motif along simulation time. The darker line denotes ATP-binding site1 (formed by Walker A motif in chain A and LSGGQ motif in chain B), and the lighter line denotes ATP binding site2 (formed by Walker A motif in chain B and LSGGQ motif in chain A).
tide binding sites and the NBD dimer. Variation of the distance between the mass centers of Walker A and the LSGGQ motif along the targeted MD trajectory (Fig. 3d) showed that the distance began to increase significantly around 150 ps.

In the ATP-bound system, the targeted MD trajectory showed that the ATP binding sites are held more tightly. The ATP molecule has extensive interactions with the Walker A motif of one subunit and uses its \( \gamma \)-phosphate to connect residues in LSGGQ motif of the opposing subunit. Because ATP molecules bring additional interactions between the NBD subunits, breaking of the nucleotide binding sites was postponed. However, this did not disturb the order of the following events as the next step would not occur until the nucleotide binding site dissociated. Throughout the targeted MD trajectory, ATP binds to the Walker A, and its interaction with the LSGGQ motif is disrupted upon the sliding movement of the NBD dimer. ADP binding at the sites hardly affected the process. Because of the absence of \( \gamma \)-phosphate, the interaction of ADP with the LSGGQ motif of the opposing NBD is weak. Therefore, the process of the nucleotide-binding site disruption is very similar with the nucleotide-free system.

**Tetrahelix Bundle at the Interface of TMD and NBD**—The conformational changes after the NBD dimer disruption took place around the tetrahelix bundle near the TMD/NBD interface. During the 4-ns equilibration run, the cytoplasmic side of TM3 and TM4 helices maintains a closely interlaced tetrahelix bundle that occludes the internal cavity from both the cytoplasm and the inner leaflet. There is an interaction network around this tetrahelix bundle near the TMD/NBD interface. There is an interaction network involving electrostatic and hydrogen-bond interactions (Fig. 4). A pair of residues on TM4 helices from the opposite TMD monomers, Glu-208\( ^{A/B} \) and Lys-212\( ^{B/A} \), contributes significantly to the packing of two TM4 helices at the cytoplasmic side. There are both electrostatic and hydrogen-bond interactions between Glu-208\( ^{A/B} \) and Lys-212\( ^{B/A} \). TM3 and TM4 helices within one monomer are connected by a pair of hydrogen bonds, Thr-121\( ^{A/B} \)-Glu-208\( ^{A/B} \) (Fig. 4). The NBDs are also involved in this interaction network.

Glu-476\( ^{A/B} \) on the x-loop of the NBD forms a salt-bridge interaction with Lys-212\( ^{A/B} \) on TM4 (Fig. 4). Upon the dissociation of the NBD dimer interface, the side chain of Glu-476 was drawn away from the tetrahelix bundle, resulting in the disruption of the two Glu-476\( ^{A/B} \)-Lys-212\( ^{A/B} \) salt-bridge interactions at about 150 and 187 ps (Fig. 3c). This step released the tetrahelix bundle from the constraints of the NBDs and facilitated the subsequent dissociation of the interaction network. It is worth noting that Glu-476 is a highly conserved residue on the x-loops among all ABC exporters (14), and cystine substitution of this residue greatly impairs the function of MsbA (53). Together with our simulation results, all of these highlight the structural and functional importance of x-loop.

After the disruption of the electrostatic interaction between Glu-467 and Lys-212, the pair of salt-bridge interactions Glu-208\( ^{A/B} \)-Lys-212\( ^{B/A} \) that connect the two TM4 helices began to break at about 228 and 295 ps, respectively (Fig. 3c). Subsequently, the hydrogen bonds and salt-bridge interactions between TM3 and TM4, such as Asp-131\( ^{A/B} \)-Gln197\( ^{A/B} \) and Thr-121\( ^{A/B} \)-Glu-208\( ^{A/B} \), dissociated at about 250 and 300 ps, respectively (Fig. 3c). So far the interaction networks used to hold the tetrahelix bundle disappeared, initiating the following large-scale rearrangements of the TM helices.

**TM Helices Rearrangement at the Cytoplasmic Side**—The intracellular helices IH1 and IH2 directly dock to the interfacial surface of the NBDs, and the Q-loops of NBD share most of the interactions with IH2. These interactions remained throughout the whole simulation trajectory. Therefore, along with the movement of the NBD dimer, IH1 and IH2 undergo large-scale displacements, rearranging the relative position of the cytoplasmic helices at the cytoplasmic side, TM3 and TM4 helices in each half of the TMD (Fig. 5, a and b). We use the distance between Thr-121\( ^{A/B} \) (TM3) and Glu-208\( ^{A/B} \) (TM4) as a measure of this movement. Fig. 5c shows that TM3 and TM4 helices begin to separate quickly from each other at about 300 ps, corresponding to the breaking of tetrahelix bundle. The separation of TM4\( ^{A} \)-TM4\( ^{B} \) and TM3\( ^{A/B} \).TM4\( ^{A/B} \) results in a portal to the central cavity opening to the cytoplasm and the inner leaflet. The splay of TM3\( ^{A/B} \)-TM4\( ^{A/B} \) helices also releases TM6 helices, which used to be tightly pinned between TM3, TM4, and ICL1. Soon after 300 ps, the cytoplasmic ends of TM6 helices together with part of ICL3 were given much more space to fluctuate. The provided flexibility likely activates the TM6 helices as the rearrangement of its periplasmic side follows. Accompanying the rearrangement of TM helices at the cytoplasmic side, TM3 and TM4 helices experienced conformational deformations transforming to a straighter form (Fig. 6a). The \( C_n \) r.m.s.d. values of TM3
and TM4 with respect to their target structures exhibited a sudden decrease after 250 ps when the tetrahelix bundle began to dissociate (Fig. 6, b and c). The conformational change of TM3 is more evident. In the initial structure, TM3 has a kink at Gly-141 in the middle of the helix resulting in a bend conformation (Fig. 6a). As the cytoplasmic end of TM3 moved toward its target position along with IH1, the helix underwent a bending deformation around a pivot at Gly-141 resulting in a straighter conformation, whereas the periplasmic half of TM3 hardly changed its position (Fig. 6a). As for the TM4 helices, as their cytoplasmic ends move apart with the dissociation of the tetrahelix bundle, the entire helices displaced from the initial positions, and at the same time the helix conformation became straighter (Fig. 6a).

**TM Helices Rearrangement at the Periplasmic Side**—The conformational transition of the periplasmic side of TMD helices includes the overall relative movement of the two “wings” and the packing rearrangement within each wing. The overall motion of the wings that closes the periplasmic side of the central pore continued throughout most of the targeted MD simulation time (Fig. 2). This motion can be monitored by the distance between the Cα atoms of Leu-52A/B on TM1 and Thr-285A/B on TM6 (Fig. 7a). Fig. 7b shows that this distance decreased linearly from the beginning of the simulation to about 350 or 450 ps. TM1A/B and TM6A/B move continually closer to each other; thereby, the two wings contract the periplasmic side to occlude the central pore.

As for the helix rearrangement within each wing, the critical step is the disruption of the interactions between TM1 and TM6 in *trans*, i.e. TM1A/B and TM6B/A. The side chains of some hydrophobic residues close to the periplasmic end of TM1A/B (Leu-48 and Leu-52), TM6B/A (Phe-288), and TM5B/A (Leu-263 and Leu-267) pack together to form a hydrophobic core in the outward-facing conformation (Fig. 7a). These interactions connect TM1A/B with TM6B/A from the opposing TMD subunits and prevent the hydrophobic side chain from fully exposing to...
the central cavity. Upon the TM helices movement at the periplasmic side, these hydrophobic interactions are disrupted, as monitored by the distance between the C\(_r\)/H\(_{9251}\) atoms of Leu-52 on TM1 and Thr-285 on TM6 in trans. Fig. 7c shows that this distance fluctuated around 7 or 8 Å in the first 350 ps and then began to increase quickly. Interestingly, some of these hydrophobic residues correspond to the substrate binding sites of the transporter. The recently reported crystal structure of MDR1 (ABCB1) in complex with cyclic peptide inhibitors revealed many drug binding residues that can be mapped on to the TM1, TM6, and TM5 helices of MsbA (8). For example, Phe-288 on TM6 and Leu-263 on TM5 correspond to Phe-974 on TM12 and Tyr-949 on TM11 of ABCB1, both of which are in common between QZ59-RRR and QZ59-SSS binding sites (8). Therefore, the disruption of the hydrophobic interactions between TM1, TM6', and TM5' likely regulates the exposure of the substrate binding sites near the periplasmic side of the transport pathway.
We noticed that this conformational movement is coupled to the changes at the cytoplasmic side. As mentioned above, the dissociation of the cytoplasmic tetrahelix bundle released TM6 helices from the packing between TM3 and TM4, enduing it with larger conformational flexibility. A possible signal transduction pathway for conformational changes is via ICL3. One end of ICL3 is coupled to the NBD subunit through the interaction pairs of Arg-333-Glu-409, Arg-333-Thr-411, and Thr-339-Asp-403, and the other end links TM6 directly and connects the elbow helix by the salt-bridge interaction of Asp-323-Arg-15. Motions of the NBD subunits might be, thus, transmitted through ICL3 up to the periplasmic side of the TM1 and TM6 helices. However, it is worth noting that the conformational changes of the two wings do not behave symmetrically among various trajectories. This could be due to the asymmetry in the initial state (structure and velocity) of the targeted MD simulations.

**DISCUSSION**

For ABC transporters, the central pore of the TMD was often found to stay in two states, i.e. the inward-facing and the outward-facing states. These two conformations were captured and characterized in many structural and biochemical studies. Although it is becoming generally believed that the transformation between the outward-facing and the inward-facing states is essential for substrate translocation and is triggered by conformational change at the NBD dimer, the atomistic details of this process remain elusive. Here, the targeted MD simulations of the MsbA exporter provide a detailed view of how the opening of the NBD dimer interface triggers the conformational
changes of TMDs and reveal the key steps during the conformational movement.

Based on numbers of targeted MD trajectories, the conformational transformation from the outward-facing to the inward-facing state can be described as successive events. It is intriguing that the order of these events depicts a clear pathway along which the conformational changes at the cytoplasmic NBD region propagate to the periplasmic side of the TMDs. In agreement with the assumption that ATP hydrolysis is the driving force for the conformational conversion, we found that the disruption of the nucleotide binding sites at the NBD dimer interface is the very first event along the targeted MD trajectories. The sliding shift movement of the NBD domains along the interface that dissociates the NBD dimer weakens the interactions between the x-loops of NBDs and the cytoplasmic tetrahelix bundle of TMD in the first place and subsequently induces the dissociation of the tetrahelix bundle by breaking the hydrogen bond and the salt-bridge interactions between TM3 and TM4 and between the two TM4 helices from different TMDs. Conformational rearrangement of the TM helices at the cytoplasmic side, especially the breaking of the tetrahelix bundle, directly results in the opening of the central cavity toward both the cytoplasm and the inner leaflet. Moreover, the conformational change at the cytoplasmic side endues the TM6 helix, which was tightly packed between TM3 and TM4 in the initial structure, with more spatial flexibility, setting out the periplasmic packing rearrangement by destroying the hydrophobic interactions between TM1, TM6, and TM5 at the periplasmic side. The signals of conformational changes at the NBD interface are, thus, transmitted sequentially through the NBD-TMD interface to the cytoplasmic side and the periplasmic side of the TMDs. The detailed picture of this process implies the functional importance of some key structure motifs and non-covalent interactions in the MsbA exporter.

At the interface of the TMDs and NBDs, the x-loop plays an important role in the cross-talk between them in addition to the Q-loop. The x-loop was recently identified as a conserved motif that precedes the LSGGGQ motif only among the ABC exporters (14). Functional studies of both MsbA and TAP demonstrated that various mutations of the conserved glutamate in the x-loop reduced or completely abolished transport activity (53, 54). Our MD simulation results showed that in the outward-facing conformation the conserved Glu-476 in the x-loop interacts with the cytoplasmic end of TM4 in cis through a salt-bridge of Glu-476-Lys-212, and this electrostatic interaction is important in pinning the NBD with the cytoplasmic tetrahelix bundle of TMD and stabilizing the tetrahelix bundle organization. The dissociation of the nucleotide-binding sites and the sliding shift movement of the NBD dimer along their interface disrupt this salt-bridge interaction, thereby initiating the following conformational changes of the TM helices. In other words, the x-loop may reinforce the stability of the outward-facing conformation by stabilizing the tetrahelix bundle, and thus, the disruption of the interaction between x-loop and TM4 affects the translocation cycle. We noticed that the Lys-212 residue in TM4 of MsbA is not conserved among other ABC exporters, such as Sav1866 or MDR1. However, in the crystal structure of the outward-facing conformation of Sav1866, the conserved Glu-473 on the x-loop forms a hydrogen-bond interaction with Val-117 on IH1 at the end of TM3 in trans, which is also in the frame of the interaction network of the tetrahelix bundle. This hydrogen bond interaction retained throughout a 4-ns MD simulation trajectory of the Sav1866 system (data not shown). Therefore, we suppose that it is a common feature of x-loops to make a remarkable contribution to the stability of the tetrahelix bundle and the cross-talk between the NBDs and TMDs.

In the outward-facing conformation of MsbA, the tetrahelix bundle is crucial for the conformational stability of TMD. It locks up the TM3 and TM4 helices at the cytoplasmic side and limits the movements of TM6 helix and ICL3 loop. Along the targeted MD trajectories, disruption of the tetrahelix bundle is directly followed by large scale packing rearrangements of the TM helices. In the outward-facing conformation, the TM3 and TM4 helices exhibit bended conformations to adapt the tetrahelix bundle arrangement and restore to the straight form in the inward-facing state. Distortions of the α-helices are also found in other transmembrane proteins, such as ion channels (55, 56) and nicotinic acetylcholine receptor (57, 58). Their flexibility is believed to be important in facilitating the conformational changes. Theoretical and computational studies showed that the bended α-helices could produce decades of piconewtons of restoring force to return to a straighter form (59). In the MsbA system, the restoring forces released from TM3 and TM4 unbending motion likely transmit to the NBD dimer through IH1 and IH2. Thus, the conformational movement of the TM helices feeds back to the NBD region, finishing the transition from the outward-facing state to the inward-facing state.

The packing rearrangement at the periplasmic side is crucial not only for the conformational change of TMD but also for the accessibility of the substrate binding site. Along the targeted MD trajectories, the disruption of the hydrophobic interactions between TM1/A/B, TM6/B/A, and TM5/B/A and the exposure of the substrate binding sites at the periplasmic side of TMD are the last happening events. This timing is likely critical for the function of MsbA exporter, as early exposure of the substrate binding site before the closure of the two wings may induce reverse transport of the substrate molecules.

It should be noted, however, that the targeted MD method has its own limitations. First, although this method allows us to explore the slow conformational transition (μs to ms) during computational accessible time scale, the relative transition progress is not necessarily proportional to actual time. A comparison between targeted MD with other perturbation MD methods, such as steered MD and biased MD methods, showed that these methods generate similar transition pathways with that of targeted MD, but the time evolution of each conformational change step may be different (60). This suggests that these perturbation MD methods can at most generate qualitatively a correct transition pathway as the free energy of the pathway cannot be evaluated directly. Second, targeted MD method cannot guarantee that the obtained trajectories follow the globally lowest free energy pathway. Application in a simple system of the alanine dipeptide showed that different initial conditions lead to statistical uncertainties in the pathway (61). Despite these drawbacks, the targeted MD method remains an attractive technique in exploring large scale conformational
Conformational Transition Pathway in ABC Transporter MsbA transition due to its computational efficiency, and many recent applications have shown that this method can provide useful information, especially in the close interplay with experimental results (62–64). In this study we found that changing initial velocities can result in differences in the details of the pathway, e.g. the time evolution of specific hydrogen bonds and salt bridges discussed above vary among different trajectories, but the order of the conformational change events is not affected. The fact that each conformational change event involves disruption and/or formation of many intra- and intermolecular interactions may enude the conformational change pathway with stability and high selectivity, although we should bear in mind that “pathway” here is to some extent “coarse-grained.”

In summary, our targeted MD simulation studies of the conformational transition from the outward-facing to the inward-facing state of MsbA exporter revealed the clear order of conformational movement steps. The disruption of interactions across the NBD dimer interface is the very first event along the targeted MD simulation trajectories, which triggers the following conformational changes. The x-loops of NBD play an important role at the interface of NBD and TMD by sensing the movement of the NBD dimer and initiating the disruption of the cytoplasmic tetrahelix bundle. Breaking of the cytoplasmic tetrahelix bundle, which is formed by TM3 and TM4 helices, induced the large-scale packing rearrangement of the TMD helices at the cytoplasmic and periplasmic sides sequentially. The packing rearrangement at the cytoplasmic side results in the opening of the central cavity toward both the cytoplasm and the inner leaflet, and the packing rearrangement at the periplasmic side exposes the substrate binding sites, which are mostly composed of hydrophobic residues. The timing of the conformational changes depicts an allosteric signal transduction pathway across the transporter from NBD to TMD and demonstrates its functional importance for the unidirectional transport of substrates.

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