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Comparison of mechanistic transport cycle models of ABC exporters

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Abstract

ABC (ATP binding cassette) transporters, ubiquitous in all kingdoms of life, carry out essential substrate transport reactions across cell membranes. Their transmembrane domains bind and translocate substrates and are connected to a pair of nucleotide binding domains, which bind and hydrolyze ATP to energize import or export of substrates. Over four decades of investigations into ABC transporters have revealed numerous details from atomic-level structural insights to their functional and physiological roles. Despite all these advances, a comprehensive understanding of the mechanistic principles of ABC transporter function remains elusive. The human multidrug resistance transporter ABCB1, also referred to as P-glycoprotein (P-gp), is one of the most intensively studied ABC exporters. Using ABCB1 as the reference point, we aim to compare the dominating mechanistic models of substrate transport and ATP hydrolysis for ABC exporters and to highlight the experimental and computational evidence in their support. In particular, we point out *in silico* studies that enhance and complement available biochemical data. “This article is part of a Special Issue entitled: Beyond the Structure Function Horizon of Membrane Proteins edited by Ute Hellmich, Rupak Doshi and Benjamin McIlwain.”

Keywords

ABC transporter; ABCB1; P-glycoprotein; Molecular dynamics simulations; Mechanistic models; Transport cycle

1 Introduction to ABC transporter function and architecture

This review discusses the architecture and function of ATP-binding cassette (ABC) exporters with a focus on ABCB1. We will present an overview of available transport cycle and ATP

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hydrolysis models, review their similarities and differences and place them into context of available data for ABCB1. Despite more than forty years of intensive research on ABCB1 and its relatives, many fundamental questions regarding substrate recognition, translocation and coupling of ATP binding and hydrolysis at the nucleotide binding domains (NBDs) to conformational changes in the transmembrane domains (TMDs) have not yet been satisfyingly resolved. Advancements of *in silico* approaches have shown great promise to complement experimental approaches and to address these questions. We will highlight available computational approaches, discuss how they were used to study ABCB1 transporter function and to test different aspects of proposed ABC transport cycle models.

ABC proteins are found in all kingdoms of life [1,2] where they mediate translocation of diverse substrates under ATP consumption. The ABC protein superfamily can be divided into four major groups, the first three of which constitute membrane proteins: (i) importers of nutrients (mainly bacterial ABC transporters) (ii) exporters of diverse compounds including peptides, metabolites or xenobiotics (such as ABCB1) (iii) ABC proteins that act as ion channels or channel regulators and (iv) ABC proteins that lack TMDs and are involved in DNA repair and translation. Most of the 48 human ABC proteins are membrane transporters (subfamilies ABCA-D, and ABCG) that facilitate directional transport of small molecules, possibly against a concentration gradient. The ABCC subfamily includes ABCC7 (CFTR: cystic fibrosis conductance regulator), which is an ion channel, as well as ABCC8 and ABCC9 that are regulators of the Kir6.2 potassium ion channel [3]. In contrast, the subfamilies E and F participate in DNA repair and translation and consist only of NBDs [4,5].

The minimal functional unit of all ABC transporters comprises two TMDs and two NBDs. Full transporters have all four domains expressed as a single polypeptide chain, while half transporters have one NBD and one TMD expressed as a single peptide chain and two of these polymers assemble to form homo- or heterodimers. All structural elements are present twice in symmetric homodimers; pseudo-symmetry is maintained (Fig. 1) in heterodimers and full transporters despite sequence divergence [6,7]. The ABCB subfamily is heterogeneous, as it includes both full length (ABCB1, ABCB4, ABCB5 and ABCB11), as well as hetero- and homodimeric half transporters (ABCB2/B3 and ABCB6-10, respectively).

For all ABC transporters, the TMDs are the site of substrate recognition and translocation; they begin at their N-terminus with the elbow helix, which is a helical structure oriented parallel to and by its amphipathic nature partially inserted into the membrane from the intracellular side (Fig. 1). Beyond their putative role in anchoring the TMDs into the membrane, in MsbA the elbow helix was also shown to interact with the substrates daunorubicin [8], suggesting a role at the beginning of the substrate translocation process.

As the ABC transporter undergoes its substrate translocation/ATP hydrolysis cycle, the TMDs go through conformational changes to bind substrate on one side of the membrane and to release it to the other. TMD conformational changes are connected to changes in the NBDs which energize the transport cycle through ATP binding and hydrolysis and induce conformational changes in the TMDs. The NBD dimer comprises two distinct nucleotide

binding sites (NBSs) [9–11], each capable of binding one nucleotide. ATP acts as the “molecular glue” that holds the NBDs together in a head-to-tail conformation. The NBSs are the most conserved parts of the protein and harbor a set of distinct motifs, such as Walker A, Walker B and ABC signature motif (C-loop), which signify ABC transporter family membership at the sequence level (Table 1, Fig. 1c). The conserved motifs are essential for nucleotide binding, hydrolysis and NBD-NBD as well as NBD-TMD communication. The NBDs can be divided into two subdomains, the core or RecA-like subdomain that includes the Walker A motif and the helical subdomain, which contains the signature motif. A plethora of *in silico* work deals with the motions of NBD dimers (Section 4) and the role of the conserved motifs. Most ABCB transporters have two canonical NBSs that bind ATP, however the heterodimeric ABCB2/B3 peptide transporter and the bile salt transporter ABCB11 contain one degenerate NBS. Non-canonical NBSs hydrolyze ATP only at a very low rate [12], because residues essential for a high hydrolysis rate deviate from the classical (or canonical) sequence (Table 1).

1.1 Short introduction to the ABCB family

Members of the human ABCB subfamily exhibit a wide variety of physiological functions. ABCB1 (multidrug resistance protein 1 (MDR1), P-glycoprotein, or P-gp) is a major player in cellular detoxification and multidrug resistance [23–25]. ABCB2 and ABCB3 (Transporter associated with Antigen Processing (TAP1 and TAP2)) form a heterodimer that is essential in adaptive immunity for loading short antigen peptides onto the major histocompatibility complex (MHC class 1) [26–28]. ABCB4 (multidrug resistance protein 2 or MDR2) [29,30] and ABCB11 (bile salt export pump or BSEP) [31,32] transport phosphatidylcholine and bile salts, respectively, into the bile duct. ABCB5 is involved in melanogenesis, the production of the pigment melanin by melanocytes [33–35]. The half transporters ABCB7 (Fe/S cluster transport) [36,37], ABCB8 (intracellular peptide trafficking) [38,39], and ABCB10 (peptide transporter) [39,40] are mitochondrial exporters. The peptide transporter ABCB9 (TAP-like or TAPL) [41–43] shows a lysosomal localization, while the cellular localization (lysosome, endo-lysosome, Golgi and/or mitochondria) and function of ABCB6 (heme and porphyrin transport) remains disputed [44–47].

The main role of ABCB1 seems to be cellular detoxification by expelling a large number of chemically unrelated hydrophobic compounds from the plasma membrane. ABCB1 is expressed at tissue barriers such as skin, intestine [48], blood brain barrier, placental barrier, and blood testis barrier [23] to limit the entry of environmental toxins and keep the level of xenobiotics below a harmful level. While normally showing a protective function, ABCB1 frequently interferes with chemotherapy treatment by actively removing the medication, when cancer cells express the transporter [49,50]. Besides its problematic role in chemotherapy, ABCB1 is of further clinical relevance since it regulates bioavailability and pharmacokinetics of many drugs, e.g. by preventing brain penetration, uptake from the intestine or permeation through the placenta [51]. ABCB1 transporter activity has also been exploited e.g. in second generation antihistamines to prevent adverse sedative effects by limiting penetration through the blood brain barrier [52].

Despite the large empirical knowledge on ABCB1 function, transported compounds and residues involved, several fundamental questions remain poorly understood at the molecular level: e.g. the molecular basis for the promiscuity of substrate recognition, the route substrates take upon extraction from the membrane through the TMDs into the extracellular space, the conformations and sequence of transport cycle states, as well as coupling between ATP binding/hydrolysis and substrate transport. Available experimental and *in silico* data will be reviewed in the following sections.

2 High-resolution views of the ABCB fold

Knowledge of protein structure is essential for understanding dynamics and function on the atomic level and to link specific interactions, conformations and structural changes to transport. Important breakthroughs in ABC transporter structural biology have been achieved: for instance, there are now structures available for human members of the ABCA [53], ABCB [54], ABCC [55] and ABCG [56,57] subfamilies. Transporters from the ABCB subfamily have been determined in three conformations: (i) most structures showed an inward open (or inward facing) conformation, where the NBDs separated by a variable range from less than a nanometer to up to a few nanometers [7,58]; (ii) The outward open conformation was found for the bacterial Sav1866 [9] and MsbA transporters [59] and showed nucleotide stabilized NBD dimers and extracellularly separated TMDs; (iii) Occluded conformations have been determined for the bacterial transporters McjD [60], PglK [61], and MsbA [62] revealing closely associated NBDs and TMDs with a fully occluded substrate binding side. Distance measurements using electron paramagnetic resonance (EPR) spin probes attached to the NBDs showed a distribution of NBD separation ranging over 2 nm in the absence of nucleotides for MsbA and ABCB1 [63,64]. The distance between Walker A motifs of ABCB1 without bound nucleotide was approximately 1.6 nm in a native lipid membrane, and 2.2 nm in a detergent micelle [65]. The addition of nucleotides induced NBD association [64,66] that resulted in a single distance peak. These data demonstrate the strong dependence of transporter conformation on the presence of nucleotides and the membrane environment and show that a large NBD separation is possible in detergents, but might be suppressed in the membrane environment. The lipid A bound cryo electron microscopy (Cryo-EM) structure of MsbA (PDB ID: 5TTP) [62], determined in lipid nanodiscs, shows an intermediate conformation in the absence of nucleotides, in which the NBDs are separated, while the TMDs are in contact through their respective C-termini. The degree and functional relevance of NBD separation is an ongoing debate in the field and has implications for ATP hydrolysis and transport models (see below).

ABCB transporters contain six core transmembrane helices per TMD that form the substrate translocation path in their center of a functional dimeric assembly. In the ABCB, ABCC and ABCD transporter subfamilies, the intracellular loops (ICLs) assemble into a tetra-helical bundle that stabilizes a long protrusion into the intracellular space (Fig. 1b), thereby positioning the NBDs far from the membrane. The transmembrane helices 4 and 5 reach over to the trans NBD in a domain swap arrangement and interact through their coupling helices with the conserved binding grooves of NBDs using a ball-and-socket type structure [9,67] (Fig. 1). A network of salt-bridges and hydrophobic interactions stabilizes the ICL

helix bundle, which has a role in transmitting nucleotide-dependent conformational changes [68], although the mechanical-functional implications of the long protrusions and the domain swapped architecture are not well understood.

Available crystal structures in different conformations show that the TMDs of ABC transporters do not move in a purely rigid body fashion during the transport cycle. Structural flexibility is encoded by helix bending, helical kinking and unwinding caused by helix breaking residues (prolines, glycines) [69]. Their mutation in ABCB1 was shown to affect coupling of the substrate translocation to ATPase activity, e.g. the G346C mutation in TM6 changed communication between the TMDs and NBDs [70], while the G185V mutation stiffened TM3 and enhanced coupling [71]. In both cases, the reduced ATP turnover and basal ATPase activity was attributed to increased helicity and an ensuing TMD rigidification.

In addition to inward and outward open conformation, transporter structures in an occluded state are available for the ABCB subfamily. These are believed to represent a transition state in the transport cycle, where the extracellular and the intracellular sides are closed, which seems to be a necessary intermediate conformation to prevent a channel like function during the transition between the outward- and the inward facing state. The known exception is ABCC7, which is an ATP dependent Cl⁻ channel. Structures of the transporters McjD [60] and PglK [61] show an occluded cavity in the center of the membrane, to which a substrate could bind. In contrast, in the cryo-EM structure of MsbA [62] the cavity is collapsed, leaving no space for binding of a lipid A substrate. It thus remains unclear, if the collapsed conformation may be part of the empty return step after substrate release and ATP hydrolysis.

Full transporters of the ABCB subfamily have a linker region of approximately 75 amino acids connecting NBD1 with TMD2 (Fig. 1a). The segment is presumably very flexible as no structural information has been obtained yet and secondary structure predictions suggest complete absence of stable structural elements. Cleavage of the linker slightly increased ATP hydrolysis rates [72], thus suggesting that it has a limited functional role. Dephosphorylation of the typically phosphorylated [73] linker decreased substrate induced ATPase activity, but maximal activity at higher substrate concentrations was maintained, suggesting that phosphorylation allows for regulation of transporter activity. Shortening of the linker by 34 residues resulted in increased basal ATPase activity in mouse ABCB1, abrogation of substrate stimulation of ATPase activity and drastically decreased transport [74]. The same linker deletion in human ABCB1 abolished function, indicating that a minimal linker length of 40–45 residues is required.

ABC transporters couple ATP binding and hydrolysis at the NBDs to conformational changes in the TMDs for substrate translocation across the membrane. Binding of substrates to the TMDs of ABCB1 increases the ATPase activity [75–77], while ATP hydrolysis and conformational changes in the NBDs lead to structural changes in the TMD [78], thus showing that the TMDs and NBDs can communicate bidirectionally. Transporter architecture implies that the ICLs and their four coupling helices are the structural elements connecting the TMDs with the NBDs and must be involved in transmitting conformational

changes. Within the NBDs, the conserved motifs of the Q- and X-loops are assumed to be essential for this interdomain communication [20,79,80]. Interestingly, in ABCB1 redundancy was found for the Q-loop, where only one of the conserved glutamines was needed for active transport [80–82], while removing both residues (Q475A/Q1118A) abrogated transport and trapped ABCB1 in the inward facing state. Involvement of these ATP interacting loops indicates that the NBS are essential elements in this process. However, while the phenomenon of inter-domain communication is well established, the precise molecular movements required for and triggered by substrate translocation and ATP hydrolysis remain unresolved. The available structures of ABCB exporters have provided a starting point for modelling and hypothesis generation for biochemical studies. Importantly, they are also essential starting structures for simulations that have been carried out to investigate several aspects of transporter function, as outlined in Section 4.

3 ABC transporter function

3.1 General models for substrate transport

The first and very general model of substrate translocation, the ‘alternating access model’ [83], proposed that a transporter alternates between an inward and an outward facing state with two different substrate affinities. Access to the substrate binding site can be locked by two gates, one of which must always be closed to prevent a channel-like function, implying the existence of an occluded transition state in which both gates are closed. The TMDs of ABCB1 assume an inward open conformation in the absence of nucleotides, with a high affinity substrate binding pocket [84]. After substrate and ATP binding at the TMDs and NBDs, respectively, the TMDs undergo structural rearrangements that allow them to first reach an occluded and then an outward open state with a decreased affinity for substrate [85,86].

Based on experimental results, the alternating access model was further developed into the ‘hydrophobic vacuum cleaner’ model [87]: (i) ABCB1 substrates were found to be structurally diverse but hydrophobic and thus to accumulate in the lipid bilayer, and (ii) spontaneous flipping of drugs between membrane leaflets is relatively slow [88]. The vacuum cleaner model requires ABCB1 to retrieve its substrate from the inner leaflet of the membrane and then to flip them into the outer leaflet or to expel them directly to the extracellular space. This model can also incorporate polyspecificity by the proposed two-step mechanism in which substrates are first enriched (depending on logP, up to several orders of magnitude) in the membrane. The high substrate concentration then allows for low affinity and thus non-selective binding. Substrates stimulate the ABCB1 ATPase activity up to ten fold [75,89–92], thereby showing that substrate binding triggers the transport activity of ABCB1. Removal of substrates enriched in the membrane allows for maintaining a low intracellular concentration in the presence of a high extracellular concentration of substrates. This model implicitly emphasizes the importance of the membrane environment for the transport process. Transport is energized by ATP, which also imposes unidirectionality [20].

3.2 Mechanistic models of the transport cycle

Models describing the steps of the transport cycle minimally include, for the NBDs, ATP binding and hydrolysis, ADP and inorganic phosphate (P_i) release and nucleotide dependent structural changes. These steps are linked to a conformational switch of the TMDs, which interchange between the high-affinity inward-facing state and the low-affinity outward-facing state. The wide opening of the TMDs as observed in the outward facing crystal structures (e.g. Sav1866 [93]) and the large separation of the NBDs seen in the inward facing crystal structures (of e.g. mouse ABCB1 [58]) have shaped the perception of the conformational changes during the transport cycle of ABCB1, though not fully consistent with all biological evidence. Many transport cycle models have been put forward which may only minimally differ in details, or might propose drastically different conformations and steps for the transport. Here, we focus only on the most recognized models. Overall, models of the catalytic cycle can be divided into two main categories; either (i) predicting complete NBD separation or (ii) requiring the NBDs to remain in contact throughout the transport cycle. Asymmetrical transporter conformations are then associated with asymmetric binding states for nucleotide. The best known models that include NBD separation are the Tweezers-like model [94], ATP switch model [95] and processive clamp model [96–98]. In contrast, the alternating sites model [99], constant contact model [100] or nucleotide occlusion model [101–103] propose continuous NBD-NBD contact of at least one NBS throughout the transport cycle. All models implicitly agree on a tight coupling between NBDs and TMDs, with the closed NBD dimer corresponding to the outward open TMD conformation and the inward facing TMD conformation showing looser NBD association. The catalytic cycle models are summarized in Table 2 and Fig. 2. There is experimental support for all these models (see Section 3.3). When considering different models, it should also be kept in mind that ATP concentrations in cells are 10-fold above K_D for ABCB1 (0.3–0.5 mM) [104], while the concentration of ADP is similar to its K_D [103]. Transporters might therefore almost always be nucleotide bound, though affinity is transporter conformation dependent [103] and still debated, as outlined below.

3.3 Experimental support for different catalytic models

The large differences in transporter architecture between type I (e.g. MalEFGK2 [105]) and II importers (e.g. BtuCDF [106]) as well as type I (e.g. ABCB1 [7]) and II exporters (e.g. ABCG5/8 [56]) seem incompatible with a single model for the transport cycle, as deduced from the mechanical implications of these structures. It also remains unresolved whether even all transporters of the ABCB family share a common transport mechanism, as they all translocate different substrates and some transporters for instance have two canonical NBS, while others harbor a degenerate site. It is well possible that degeneration of one ATPase implies a change in the mechanism of harvesting the energy from ATP binding and hydrolysis and thereby leading to an alternative route through the transport cycle. Interestingly, a comparison of supporting experimental evidence reveals that switch and processive clamp models are mainly supported by measurements on bacterial transporters. In contrast, the alternating sites, constant contact and nucleotide occlusion models are mainly supported by data from mammalian transporters. It remains unresolved, if these differences i) represent a common trend with the bacterial and mammalian transporters using different

mechanisms, ii) are due to experimental underdetermination, thereby leaving room for interpretation, or iii) can be attributed to the particularities of each transporter.

3.3.1 Asymmetric NBS occupation—Un-synchronized ATP binding or hydrolysis lead to asymmetric nucleotide binding states in the NBSs, which may induce structural and functional asymmetry for the entire transporter. Asymmetric conformations are predicted by the alternating sites, the nucleotide occlusion and the constant contact models. Asymmetric conformations may seem expected for ABC transporters with a non-canonical NBS, but for transporters with two canonical, fully ATPase competent sites, structural symmetry could be preserved. Models supporting an asymmetric mechanism like the constant contact model [107] emphasize relative motions of the helical vs. the core (RecA-like) sub-domains within the NBDs. This extra flexibility would allow nucleotide exchange in the open NBS, while keeping the second NBS closed and ATP bound in an asymmetric conformation [107]. The coupling helices of the TMDs are attached to the NBDs at the boundary between the core and helical domain, suggesting that communication between the sub-domains could allow for cross-talk to the TMDs by allostery. Moreover, intra-NBD interactions between motifs and nucleotides (e.g. the formation of the catalytic dyad in the closed NBS [22]) have been used to explain the cooperative nature of ATP hydrolysis.

Functional asymmetry and differences in affinity for ATP γ S (a non-hydrolyzable ATP analogue) to both NBS have been determined for Chinese hamster ABCB1 and revealed two very different KD values of $6 \pm 4 \mu\text{M}$ and $740 \pm 420 \mu\text{M}$ depending on the occlusion of the NBS [103]. An asymmetric intermediate was captured by trapping Chinese hamster ABCB1 in the post-hydrolytic state by replacing the inorganic phosphate (P_i) with orthovanadate (V_i) leading to a $\sim 1:1$ stoichiometry, i.e. ATP hydrolysis in one NBS resulting in a very stable transporter conformation [75]. In contrast, in bacterial ABC exporters, the pre-hydrolytic trapped state by BeF_x [108], the V_i trapped state with Mn^{2+} (replacing Mg^{2+}) [109] and the coordination of Mn^{2+} in the ATP-bound state were observed to be symmetric [110]. Mutation of the Walker B catalytic glutamate also trapped the protein in an asymmetric state with tight binding of a single ATP to one of the NBSs and referred to as nucleotide occlusion, while the second NBS took a more open conformation with a lower ATP affinity [103,111]. From the asymmetric ATP trapping it may be inferred that ATP hydrolysis is most likely not synchronized between the NBSs of ABCB1. However, the functional implications for the number of ATPs hydrolyzed (see below) remain unresolved, because asymmetric nucleotide trapping with V_i is consistent with a single ATP hydrolysis as well as with two sequential ATP hydrolysis events per transport cycle.

3.3.2 Number of ATP hydrolyzed per cycle—Despite numerous studies, it is still debated whether hydrolysis of one or two ATPs is needed for a transport event and different experiments showed different outcomes (see e.g. [81,101,112–114]). In the NBD dimer of the yeast intracellular peptide transporter Mdl1p, ATP hydrolysis of one ATP is followed by formation of a stable intermediate with one ATP and one ADP bound, which then allows for hydrolysis of the second ATP. However, under ATP limiting conditions, the dimer dissociates before hydrolysis of the second ATP [96]. Similarly, in the bacterial NBD-only system MJ0796, the NBDs dissociate after hydrolysis of one ATP [115] suggesting that a

single ATP hydrolysis event is sufficient for NBD separation. In case of mouse ABCB1, single Walker B catalytic glutamate mutants were found to still allow for conformational changes, while the double catalytic mutant trapped the transporter in the outward facing conformation [66]. Of note, transporters with a degenerate NBS (e.g. bacterial TM287/288, or mammalian ABCB2/B3 and ABCB11 as well as all transporters of the ABCC subfamily) are able to support transport with only one canonical NBS, suggesting that a single ATP hydrolysis event can in principle suffice for sustaining transport.

3.3.3 NBD separation—Experimental results suggest that two ATPs bound to the NBDs are needed for complete NBD dimerization, which agrees with all models [96,114,116]. However, it still remains debated, if the NBDs fully separate during the transport cycle. Widely inward open crystal structures of eukaryotic ABCB1 suggested that this can indeed be the case [7,58,74]. Similarly, EPR, lanthanide-based resonance energy transfer (LRET) and cryo-EM data on bacterial ABC transporters showed that their NBDs can undergo full separation in the absence of nucleotides when purified in detergent micelles [66,116,117]. In spite of the observed separation between the NBDs in ABCB1, cross-linking experiments showed that human ABCB1 cross-linked at its ICLs (L175C/ N820C) remained ATPase active [118] and restored ATPase activity of a Q-loop double mutant Q475A/Q1118A [82], suggesting that ATPase activity does not per se require a wide separation of NBDs.

It is very difficult to quantify the transport cycle with sufficiently high temporal and spatial resolution. Answering the questions discussed above, i.e. NBD separation, number of ATP hydrolyzed per translocation event, symmetry for transporter conformation and nucleotides occupancy should enable clarification of the essential steps of the power stroke of the transport cycle. Simulations can in principle address these questions by enhanced sampling methods, but rely heavily on empirical input from models and experiments (for details, see Section 4). While challenging, a combination of simulations and experiments with repeated hypothesis generation and testing should be able to explore the framework provided by the transport models and further define the transport cycle.

4 In silico assessment of substrate transport and ATP hydrolysis

To fully understand substrate recognition or conversion of chemical energy stored in the ATP phosphate bonds to conformational changes that lead to substrate transport, insights into the structural, mechanical, dynamic and energetic properties are needed throughout the transport cycle at the atomic level. Experimental approaches, despite their impressive advancements, have limitations: EPR and FRET-based approaches for instance yield information about protein structure and dynamics, but rely on probes added to specific positions within the protein, and require protein modification. X-ray crystallography yields high-resolution all-atom snapshots of proteins, but requires crystal formation. Cryo-EM can obtain near atom resolution structures, but requires averaging of many images and sorting of particles. Molecular dynamics (MD) simulations are very useful for structural and dynamic investigations at the atomic level and can in principle provide detailed information on conformations, dynamics, populations, energies, barriers and allostery for the entire transport cycle. They are therefore well placed to complement and enhance wet lab experimental approaches. Classical MD simulations use a force field to describe matter. A

force field consists of a collection of functions combined with empirical parameters tailored to optimally approximate the underlying chemical and physical principles without the need of solving computationally extremely expensive quantum mechanical equations. Force fields include bonded terms (bonds, angles and torsions) that describe the interactions through covalently bonded atoms, as well as non-bonded through-space electrostatic (Coulomb) and van der Waals interactions. MD simulations solve Newton's equation of motion. They start with a system consisting of a defined number of atoms and molecules in an initial configuration and explore the dynamics of these atoms and their motions through the underlying energy landscape (or hypersurface). Temperature is represented by atom velocities at the molecular level and thus connected to energy barriers and to the extent of conformational changes that can be achieved. Quantification of free energy profiles of paths connecting different states become accessible when all relevant conformations are visited with a frequency that corresponds to the underlying free energy profile, thereby providing Boltzmann distributions.

For the study of protein function, high resolution structures with representative conformations are an essential input, because non-representative or high energy conformations can lead to un-physiological conformational changes and structures. Even when a high resolution, membrane embedded protein structure is available, MD simulations are no free lunch. A single time step (the time resolution of the simulation) is limited to 2 fs, thus 2 μ s of simulation time require 1 billion repeating steps of solving Newton's equation of motions for all particles. Sampling, which is the exploration of the energy landscape to reach representative conformations and populations, requires massive computer time, which is only available on dedicated hardware or large high-performance computer clusters. The time windows that are now accessible for investigations with atomic details are in the microsecond range, which still falls orders of magnitude short of the time required for completing the transport cycle of ABCB1. While ATP hydrolysis happens in the millisecond timescale within the NBDs [119], the full transport cycle is a fraction of a second – a timeframe that is currently unattainable with unbiased MD simulations [120–122]. Fig. 3 shows time scales of biological processes relevant for transport by ABC proteins and compares these to simulation times accessible with different MD techniques (see below).

The sampling limitation can be amended by non-equilibrium methods and biased techniques. Bias or external forces can be applied to accelerate the search in conformational space or to limit the exploration to the interesting low energy regions [123–126]. This allows to investigate the low energy conformations (e.g. metadynamics [124]) or to push the system across energy barriers (e.g. by steered MD [126]). These methods can keep track of the bias and use this information to reconstruction of the original underlying free energy landscape by postprocessing analysis or to estimate the work (or activation energy) needed for a conformational transition. Reaction coordinates represent the interesting degrees of freedom and need typically to be defined beforehand. They can be very general, consisting of a simple linear distance between two atoms, or very complex geometric operations describing domain rearrangements and conformational changes. Reaction coordinates can also represent a chemical reaction, e.g. ATP hydrolysis. Definition of reaction coordinates remains a challenge and a deep understanding of the molecular mechanism and the

conformational changes are required, which is often not known at the necessary level of detail.

Another way to speed up simulations is to decrease the number of particles used to explicitly describe the system. This can be obtained by merging three or four atoms into a single larger virtual particle using a technique called coarse graining, but requires the time-consuming development of a new tailored force field [127]. A speedup of 10,000–40,000 [128] is achievable and puts the millisecond time window at reach. This performance boost is possible, because the calculation time step is extended from 2 fs to 20–40 fs, the number of calculated interactions is decreased by 10-fold and the energy hypersurface becomes much smoother. Coarse graining is a very successful model for membrane dynamics and recapitulates most membrane properties [129], but its application to proteins [130] is limited, as hydrogen bonds, the peptide bond, protein secondary structure or structural changes cannot be fully described. The coarse grained Martini force field was successfully applied to study protein-lipid interactions, protein diffusion and protein aggregation [131,132]. It can be expected that coarse grained simulations will become a widely used technique for the study of interactions of membrane components such as cholesterol or substrates with membrane proteins including ABC transporters.

Several focused questions regarding ABCB1 (and homologues) function have been addressed with simulations, revealing details of the transport cycle (discussed below). Simulations of the large and slowly moving ABC transporter systems need experimental data to develop reaction coordinates for enhanced sampling approaches, which are needed to ameliorate the sampling challenge (as outlined above). However, incorporation of experimental data can be challenging due to the diversity of models proposed for the ABC transport cycle and hence the requirement to translate these into different reaction coordinates required for enhanced sampling techniques. In addition, the frequently observed inward facing transporter structures have been difficult to use as starting structures for ABC transporter simulations. Strikingly, they appeared to be relatively unstable when inserted into membrane [133], which makes it difficult to discriminate between interesting conformational changes and the initial relaxation of the starting coordinates during the equilibration phase of any simulation. Despite these challenges, a number of interesting studies have been put forward that investigate ABC transporter/ABCB1 function and dynamics with *in silico* approaches:

4.1 Dynamics of the nucleotide binding domains

A key distinction between the two main types of transport models is the degree of NBD separation during the transport cycle. MD simulations should be able to identify the correct model by exploring conformations, dynamics and energetics of NBD separation. Several MD simulations of full length ABC transporters have been carried out starting from the inward or the outward facing conformations, thus beginning to elucidate structural changes and their dependence on the nucleotide bound state.

The NBD dimer stabilizing effect of ATP binding to the Walker A and ABC signature motifs is well characterized and accepted [66,134–139]. A driving force for NBD association is charge complementation: Walker A and the ABC signature motif are both positively charged

on the NBS surface, and structurally in close proximity within each NBS, but sequentially located on opposing NBDs, therefore repelling each other in the absence of nucleotides. Binding of ATP, which carries a charge of - 4, complements their electrostatic fields and exerts an attractive force on both NBDs, thereby promoting NBD dimerization.

Simulation of full length transporters (Sav1866, MsbA, ABCB1) starting from a closed NBD conformation [79] [136] indicate that two ATPs are necessary to maintain the outward open conformation and the closed NBD dimer geometry, which is in agreement with crystal structure showing two bound nucleotides in the Sav1866 and MsbA crystal structure [59,93]. The NBDs of ATP-bound ABCB1 remained associated, but became asymmetric in the presence of two ATP and Mg^{2+} [136]. In simulations of Sav1866, bound ATP stably maintained the symmetric dimer conformation [140]. For Sav1866, during simulations of the posthydrolysis state, neither ADP nor Pi dissociated from the NBDs over the course of sub-microsecond long simulations [140]. Opening of the TMD intracellular gate was reported, while closure of the extracellular side was not contemporaneously observed. However, it seems unlikely that this is a stable conformation, as a structure with both gates in the open conformation would represent a channel. Simulations of asymmetric nucleotide bound states will be discussed in the next section. In contrast, in the absence of nucleotides, the Sav1866 NBD-NBD interface became more mobile and hydrated, but did not dissociate, while closure of the outward open TMDs conformation was seen [141]. These results suggest that NBD separation might not be necessary to reach an occluded conformation. Comparable conformations were also observed in the crystal structures of McjD [60] and PglK [61]. This indicates that an occluded TMD conformation is plausible when the NBD are closely associated.

MD simulations studying nucleotide induced NBD associations were also carried out and started from inward facing conformations. MD simulations of the *T. maritima* TM287/288 heterodimeric exporter indicate that a single bound ATP is not enough to promote dimerization [134] as two nucleotides were needed to induce dimerization. Consistently, EPR data [137] showed that TM287/288 populates both inward- and outward facing conformations at hydrolysing conditions in the presence of ATP, while when trapped by V_i or by mutation of the catalytic glutamate, an associated NBD dimer geometry was observed, showing that the presence of nucleotide is required. The same conclusion was reached for the bacterial transporter MJ0796 (from *M. jan-naschii*) by LRET examining the association behavior of isolated NBDs [116].

Simulations starting from full-length ABC transporter structures with larger NBD separation turned out to be more challenging. Starting structures of MsbA [142–144] and ABCB1 [69] resulted unstable and changed conformation towards smaller NBD separation. Thereby, the structures spontaneously moved along a naturally existing energy gradient towards a lower energy state, which is a shallow but wide energy basin leading to very dynamic conformations, in line with EPR data [144]. For simulations starting from the inward open mouse ABCB1 structure [145] inserted in a lipid bilayer membrane, two metastable states could be reached in the presence of ATP: either i) ATP interacted only with the corresponding Walker A motif or ii) it also connected with the ABC signature motif of the opposite NBD, forming a partial dimer [121]. A canonical dimer structure was not observed,

possibly due to the absence of substrate [121], or alternatively, sampling might have been insufficient for reaching a canonical dimer geometry in these unbiased simulations.

In summary, simulations suggest that bacterial homodimeric ABC transporters might reach the closed NBD dimer in a symmetric conformation in the presence of two bound ATPs. These results are in line with the ATP switch or the processive clamp model, which include/imply symmetry in the ATP bound state, but also indicate the presence of fully separated NBDs after ATP hydrolysis. In contrast, the results from simulations of ABCB1 are less well converged, therefore not being able to rule out that the observed asymmetry could also be the result of limited sampling or the consequence of unstable (and therefore nonequilibrium) starting structures (models).

4.2 Asymmetric nucleotide binding and hydrolysis

Structural asymmetry in the NBDs has frequently been observed in simulations using an asymmetric nucleotide binding configuration. Opening of NBSs was seen when devoid of nucleotide, in line with most transport cycle models which predict that a closed, but empty NBS should not exist. A simulation starting from the symmetric Sav1866 transporter structure, while containing only one ATP, showed opening of the apo site and in parallel a further reduction of the Walker A - signature sequence distance in the ATP bound NBS [146], when compared to the crystal structure. Similar observations were made in simulations of Sav1866 with ATP in both NBSs or with ATP in NBS1 and ADP in NBS2. Here, ATP maintained strong interactions between the NBDs, while ADP could not stabilize the NBS bound geometry, leading to asymmetric NBS opening [147]. At the same time, the interactions within the tetra-helix bundle of the ICLs became weaker and increased flexibility was apparent in both NBSs. Closure of the TMD extracellular region was observed in one of the ATP + ADP bound replicas accompanied by twisting motions of the NBDs, suggesting that a NBD dissociation might not be necessary for reaching the closed TMD conformation, which is in line with alternating sites and constant contact models. In contrast, experimental data on bacterial ABC exporters, such as MsbA and LmrA showed that the NBDs are very mobile in the absence of nucleotide, and separate by over 2 nm [63,64,148], in line with full NBD separation during the transport cycle.

Another important distinction between ABC transporter models are the number of ATP molecules hydrolyzed per transport cycle. This has implications for mechanical coupling between ATP hydrolysis and transport. Binding of ATP to an NBS exerts an attractive force on both NBDs, whereby the γ -phosphate ($P\gamma$) predominantly forms hydrogen bonds with the ABC signature sequence, while the $P\alpha$ and $P\beta$ mainly interact with the Walker A and B motifs. Breaking of the bond between $P\beta$ and $P\gamma$ by ATP hydrolysis leads to a dramatic change in the equilibrium of forces. Now, the connection between the NBDs in the form of the ATP phosphate chain no longer exists, because ADP and P_i are two separate molecules. Importantly, within the NBDs, the NBSs are symmetrically arranged, but displaced from the geometric center. Thus, hydrolysis of a single ATP in one NBS will lead to rotational forces promoting asymmetric opening of one NBS, unless ATP hydrolysis is synchronized between the two NBSs. Synchronized hydrolyses of both ATPs would not generate such torque as both NBSs exert equal and therefore symmetrically balanced forces leading to linear NBD

separation without rotation. Asymmetric models, which require only one ATP hydrolysis event to proceed through the transport cycle, therefore also need to include NBD rotation. In contrast, the ATP switch and the processive clamp models predict hydrolysis of two ATPs. Hydrolysis might then be synchronized, or proceed in two sequential steps, which leads to a new challenge: in the former situation, a mechanism is required for synchronized hydrolysis. In the latter, the symmetric complex (which is a high energy configuration [66,149]) needs to retain the first ADP + P_i until the second ATP is hydrolyzed or alternatively, energy conversion might proceed through two sequential rotational steps with opposing rotational direction. One of the supporting arguments for a two-step ATP hydrolysis mechanism is that both NBSs of ABCB1 are equally capable for ATP hydrolysis and that ABCB1 activity is fully blocked when one site is degenerated by a mutation [150]. In contrast, for ABCB11 a single ATP hydrolysis event might suffice, because it has only one canonical NBS which can hydrolyze ATP at a sufficient rate to sustain transport.

In summary, simulations of ABCB1 and its homologues have shown that the geometry of the NBDs are sensitive to the state of nucleotide binding, showing that ATP stabilizes the bound state, while ADP, ADP + P_i or the apo NBS induce separation, thereby recapitulating and complementing experimental results. Difficulties arise from limited sampling or unstable starting conformations, which need to be overcome to discriminate between the various transport models. There is accumulating (but not yet fully conclusive) support to indicate that bacterial homodimeric transporters might largely follow geometrically symmetric steps through the transport cycle. In contrast, the asymmetry observed in several ABCB1 simulations indicates that ABCB1 possibly follows a largely asymmetric path through the transport cycle.

4.3 Molecular mechanism of ATP hydrolysis

ATP binding and hydrolysis are processes that involve most of the conserved motifs within the NBD (Fig. 1c and Table 1): Conserved motifs such as A-loop, Walker A and ABC signature motif stabilize ATP, while the Mg²⁺ ion is positioned by ATP supported by the Q-loop and the Walker B glutamate. Both the Walker B glutamate and the H-loop histidine are necessary for effective ATP hydrolysis [22,151]. The first proposed catalytic mechanism was a ‘general base model’: the Walker B glutamate first accepts a proton from the lytic water molecule. The resulting hydroxide ion then attacks the γ -phosphate of ATP, which results in P β O-P γ bond breakage and subsequent P_i release [152–154] (Fig. 4). The ‘general acid model’ assumes that a proton is abstracted from the H-loop histidine by P γ of ATP, which in turn removes a hydrogen from the lytic water. The resulting hydroxide ion can then attack the P β O-P γ bond of ATP. As a third alternative, the ‘substrate-assisted catalysis’ mechanism is built on the role of the H-loop histidine, which was proposed to maintain the conformation of the catalytic dyad, while water spontaneously attacked the P γ of ATP [22].

Recently, ATP hydrolysis in the NBDs (MalK2) of in the Maltose transporter MalEFGK₂ was directly investigated using a mixed quantum mechanics/molecular mechanics (QM/MM) simulation approach. The atoms involved in the chemical reaction itself were described by a computationally very expensive quantum mechanical (QM) treatment [155] to describe chemical reactions of bond formation and bond breaking. This inner QM core of

the simulations was then surrounded by all other atoms of the system described at the molecular mechanic (MM) classical force field level, which are orders of magnitude less computationally expensive. The three ATP hydrolysis scenarios were tested by combining the QM/MM simulations with a metadynamic biased sampling technique to steer the exploration of the energy hypersurface along the preset reaction coordinates of each hydrolysis model. The calculations showed the lowest activation barrier for the general base catalysis mechanism (43.9 kJ/mol), followed by the general acid catalysis mechanism (92.5 kJ/mol), while the substrate-assisted catalysis mechanism would require 134.3 kJ/mol. Experiments found an activation energy of 72.3 ± 4.2 kJ/mol [156] (Fig. 4). These results show that QM/MM approaches have the potential to reveal individual steps of the ATP hydrolysis event with atomic resolution.

5 Global conformational changes throughout the transport cycle

The ABC transporter transmembrane domains form the substrate translocation path across the membrane. The substrate binding pocket is of higher affinity for compounds, when accessible from the intracellular side and of lower affinity when exposed to the extracellular side [86]. The conformational changes of the transport cycle lead to changes between the substrate translocation path and also the residues constituting the substrate binding site explaining the changing affinity. The conformational transition between outward and inward facing states of MsbA was simulated, where the overall work and the reaction path were optimized to develop a low energy and therefore representative transition path [142,143]. As the protein is pushed from one conformation to the other along reaction coordinates, energy input (i.e. work) is required. Since this work is path dependent, it is presumed that paths requiring the least work are the most representative. The selected reaction coordinates were TMD angles, NBD center of mass distance and angle and were applied symmetrically to both half transporters. The least work was needed when the transporter was transformed from the outward to the inward open conformation in four distinct transition phases: (i) closing the periplasmic gate of the TMDs, and (ii) twisting the NBDs. The NBD rotation was suggested to be a necessary component of the conformational transition that has to twist before the cytoplasmic gate can be opened. The NBD twist was followed by (iii) increasing the NBD-NBD distance and finally (iv) opening the substrate binding pocket towards the intracellular side (see [142,143] for details).

TMD flexibility was found to decrease upon binding of ATP to the NBDs of ABCB1 in MD simulations [136], in agreement with experimental observations [66,137,157]. Similarly, MD simulations showed that the outward closed conformation of the ABCB1 was stabilized by bound ATP [79]. This finding was unexpected, because it had been generally assumed that the TMDs reach the outward open conformation upon ATP binding and NBD dimerization, as observed e.g. in the structure of Sav1866 [9]. However, the crystal structures of McjD [60], MsbA [62] and PglK [61] also showed an occluded TMD conformation in the presence of nucleotides and dimerized NBDs. Based on the MD simulations of ABCB1 [79], it was speculated that the ATP hydrolysis step, rather than ATP binding, might push the TMDs from the outward closed to the outward open conformation. These findings are supported by experimental results from EPR measurements on ABCB1 [66], while EPR data on MsbA

[144], LmrA [63], ABCB1 [62] and structures of the bacterial ABC exporters Sav1866 and MsbA [59,93] showed outward open structures in the presence of AMPNP.

The study of the full transport cycle, which takes approximately 0.3 s for ABCB1, requires enhanced sampling techniques and new methodological developments as applied for the study of MsbA [142,143]. Computational studies revealing the dynamics of the transport process at all atom resolution will remain a challenge, because of (i) the large difference in the time window (simulations now reach low μ s, while the transport cycle is in the range of hundreds of milliseconds), (ii) the complexity of the conformational changes requiring extensive exploration of conformational space and (iii) the uncertainty of the sequence of steps implies the comparison of multiple paths. Simulations of the transport cycle will continue to rely on the guidance from experimental data to be able to address these challenges and to contribute a dynamic all atom view.

5.1 Membrane effects

Conformation and function of membrane proteins is strongly dependent on their environment, the lipid bilayer. Since many ABC transporters recruit their cargo from the membrane, with ABCB1 being a prime example, such a dependence on the lipid environment may be exceptionally pronounced. As recognized early on, compounds interacting with ABCB1 are typically lipophilic or amphipathic and accumulate in the membrane [18]. It was observed that, due to this accumulation, the measured affinities for substrate binding from the membrane are in the millimolar range [84,85,158,159]. This may also have facilitated the development of polyspecificity, as observed in ABCB1 (and other multidrug transporters), because the typically high enthalpic component of the binding energy, which also leads to high specificity and selectivity, is not needed and is possibly low. This expected small contribution of enthalpy to substrate binding is a burden for docking studies, because scoring functions used in docking are optimized for high affinity binders and largely rely on the enthalpic contribution of binding.

Beyond accumulating substrates, the membrane environment plays a second important role by exerting mechanical and structural constraints on the transporter. These are caused by lateral membrane pressure [160,161], membrane thickness [162] and fluidity [163], all of which are important for transporter stabilization and functionality. Experiments showed that ABCB1 activity can be modulated by changes in membrane thickness [117,159] and fluidity, membrane melting temperature [164], membrane composition [75,117], while detergents reduced or possibly eliminated ATPase activity [159]. Loo and Clarke [82] observed that ATPase and transport function of ICL cross-linked mutant transporters differed between native membrane and liposomes. We would like to refer our reader to the detailed summaries on the topic of experimental approaches studying lipid/membrane dependency of ABCB1 [165,166]. The effects of the environment on ABCB1 have also been addressed by MD simulations. Here, the conformational behavior of ABCB1 in conditions resembling the ionic detergent sodium cholate conditions used for crystallization were compared with a membrane environment containing cholesterol, POPC (palmitoyl-oleoyl-phosphatidylcholine) and 150 mM NaCl [133]. Interestingly, the transporter remained close to the conformation observed in the crystal only if the environment also resembled the conditions

used for crystallization. In contrast, ABCB1 showed large conformational changes in the membrane environment leading to a decrease of the NBD-NBD distance. Similarly, LRET studies on MsbA observed differences in NBD separation based on the membrane environment [119]. Such effects can potentially be rationalized by comparing the highly dynamic and adjustable detergent environment of a micelle with the membrane environment that forms a two-dimensional fluid imposing constraint.

5.2 Substrate binding

The TMD dimer in ABCB1 contains highly promiscuous binding site (s) for substrate and forms a translocation path in its center (Fig. 5). The interaction of the TMDs with substrates has been thoroughly investigated, and many residues involved in ligand binding were identified [167–169]. Biochemical analysis showed that all transmembrane helices are involved in substrate binding, covering a volume within the TMDs that is larger than any known ABCB1 substrate. ABCB1 is known to bind at least 300 compounds [170] and distinct binding sites for Hoechst 33324, rhodamine 123 [171] and prazosin [76] have been mapped. ABCB1 substrates and inhibitors have apparent affinities ranging from 37 nM to 160 mM [166], with compounds inhibiting ABCB1 showing a slightly higher affinity [135,172]. Binding is dominated by unspecific hydrophobic interactions, but specific hydrogen bonds have also been identified [173]. The residues identified to be involved in substrate interactions are oriented towards the inner cavity of the TMD and cluster halfway through the membrane, close to the restriction area as apparent in the crystal structures of inward facing ABCB1 (Fig. 5) [167,169,174]. Binding of the ligands QZ59-RRR and QZ59-SSS to ABCB1 in crystal structures (PDB ID: 4M2S and 4M2T) [58] and of lipid A to MsbA (PDB ID: 5TV4) [62] in a cryo-EM structure has been observed. The substrate binding sites overlap to a large extent with the biochemically identified ligand binding sites as highlighted in Fig. 5. Polyspecificity is attributed to the abundance of hydrophobic residues in the binding pocket, which provide a large and mainly hydrophobic patch for binding of various compounds. These residues adjust their conformation upon transition to the outward facing conformation, thereby leading to a decrease in affinity [74,168]. Free energy calculations were used to study ligand binding and to identify the binding site of nifedipine and morphine to inward facing ABCB1 [175]. These calculations showed an energy minimum for substrate binding when interacting with residues known to be involved in ligand binding. Enhanced sampling methods were used to study the substrate transport event of ABCB1 by inducing a transition from the inward to the outward facing state. Daunorubicin and verapamil were then found to move over 1 nm towards the extracellular compartment [176]. Substrates did not show specificity for one substrate translocation path, when comparing repeated simulation, suggesting that the process could be stochastic [172]. In contrast, functional data indicate that ABCB1 might contain two at least partially separated translocation paths [173,177], because substrates (rhodamine 123, verapamil, propafenone) associated with the R (for rhodamine binding) and the H (for Hoechst binding) site [171] were differently affected by charged residues positioned within the substrate translocation path.

Promiscuity and weak binding are challenges for experimental as well as computational approaches. For correct *in silico* treatment, all ligand binding poses and their binding energy

must be known. The hydrophobic nature of ABCB1-drug interactions requires treatment of entropy at high precision, which implies extensive sampling to obtain Boltzmann weighted distributions. With the availability of massive computer resources, it should now become possible to identify those properties that characterize e.g. the R and the H site.

6 Conclusions and outlook

Although many details of substrate recognition and translocation, ATP binding and hydrolysis as well as ABC transporter structures and structural changes have been elucidated, the mechanism of the overall transport cycle of ABCB1 remains elusive at the molecular level. TMDs and NBDs are linked by structural cross-talk as substrate binding increases the ATP hydrolysis rate and ATP hydrolysis leads to substrate translocation. The ICLs and some NBD motifs seem to have an essential role in this allosteric coupling. The NBDs form a sandwich dimer upon ATP binding, while the NBSs open to an uncertain extent after hydrolysis. Changes in the NBD-NBD conformation (distance and/or orientation) allow for the conversion of the chemical energy of ATP into mechanical energy by structural dynamics and conformation changes, also ensuring unidirectionality of transport. Environmental factors have an additional impact on substrate transport by ABC transporters. The membrane can increase the local substrate concentrations by several orders of magnitude; hence a high affinity substrate binding site might not be necessary to support the barrier function of ABCB1. The membrane also provides mechanical and structural support by affecting transporter conformation, conformational equilibria, confining motions and consequently impacting on the height of energy barriers encountered throughout the transport cycle. Increasing experimental and computational data suggest that the widely inward open conformations captured by some crystal structures might be too extreme, while some seemingly contradictory observations (e.g. differential effects of nucleotide binding and hydrolysis and NBD closure as observed by spectroscopic techniques) await elucidation.

Accumulating data suggests that the transport cycle of ABCB1 includes asymmetric states and that the NBDs carry an intrinsic tendency to adopt an asymmetrically occluded conformation. A similar asymmetry has until now rarely been observed in bacterial (homodimeric) half transporters. The available information still leaves room for both main types of models of the catalytic cycle (with/without NBD separation). It has also not yet been conclusively answered, whether all ABC transporters from the ABCB, ABCC and ABCD subfamilies, from archaea to humans, follow the same path through the transport cycle, especially because some transporters have two canonical NBS that efficiently hydrolyze ATP, while others carry a degenerate site. Consensus exists that ATP brings the two NBDs together. It remains debated however, whether the concentration of ATP, which in the cytosol is tenfold above K_D , suffices to continuously bind and maintain ABC transporters in the ATP-bound conformation; this question is intimately linked to how far the NBDs can separate when the transporter is in its native membrane environment. The importance of the membrane in affecting protein conformations and equilibria is often underestimated, maybe because it is so difficult to quantify. For simulations starting from detergent solubilized transporter crystal structures, these effects are of pivotal importance and need to be correctly addressed to draw conclusions. The use of more native like

environments in experiments, such as nanodiscs in cryo-EM or lipid cubic phase in crystallographic studies, is expected to improve the situation in the near future.

Several *in silico* studies provide important observations of the conformational changes of ABC transporters. Knowledge of forces and energies will be required to fully comprehend ABCB1 function from simulations. Structurally encoded dynamic movements of NBDs and TMDs have been captured. The next important step will be to connect NBDs and TMDs movements to nucleotides and substrate interactions with the aim to study substrate translocation directly as well as to elucidate the cross-talk between these domains. Computational studies have the advantage to contemporaneously provide dynamic information for all atoms, but also carry substantial limitations. In contrast, experimental data are often coarser, frequently exclusively probing e.g. one specific interaction or distance with high precision, but typically do not suffer from sampling problems. Building on the high complementary of obtainable results, it will most likely be a well-placed combination of several methods that can assemble and add the last piece to the tricky puzzle of the transport cycle of ABC transporters. *in silico* approaches are clearly part of the method portfolio to shed light on the remaining ABC transporter mysteries and to push our understanding of ABC transporter biology beyond the structure-function horizon.

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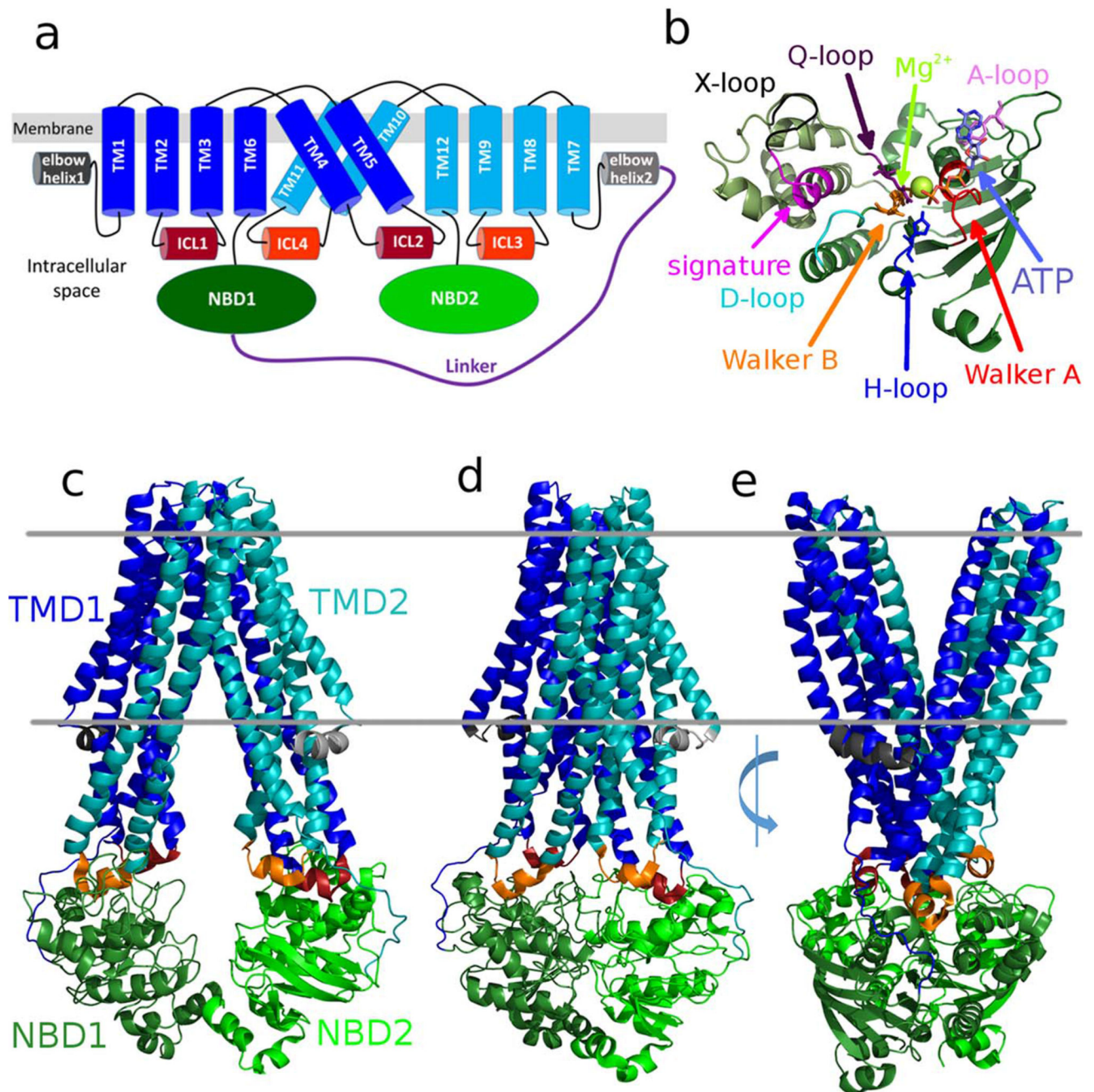


Fig. 1. Structural features of ABCB1.

a) Topological model of the ABCB fold. The elbow helices preceding TMH1/TMH7 anchor the TMDs in the membrane, the long ICL2/4 cross-over and interact with the trans-NBD, while ICL1/3 bind to the cis-NBD. b) Focused view on the nucleotide binding domain looking at the NBD-NBD interface, highlighting the conserved motifs with bound Mg²⁺ and ATP. Crystal structures of c) inward open, d) occluded and e) outward open conformation of MsbA (PDB ID: 5TV4, 5TTP, 3B60, respectively) colored according to the topological model. The inward open structure shows separated NBDs, the occluded structure is closed

on the intracellular and the extracellular side. The outward open structure shows a large separation of the TMDs towards the extracellular space allowing for substrate release.

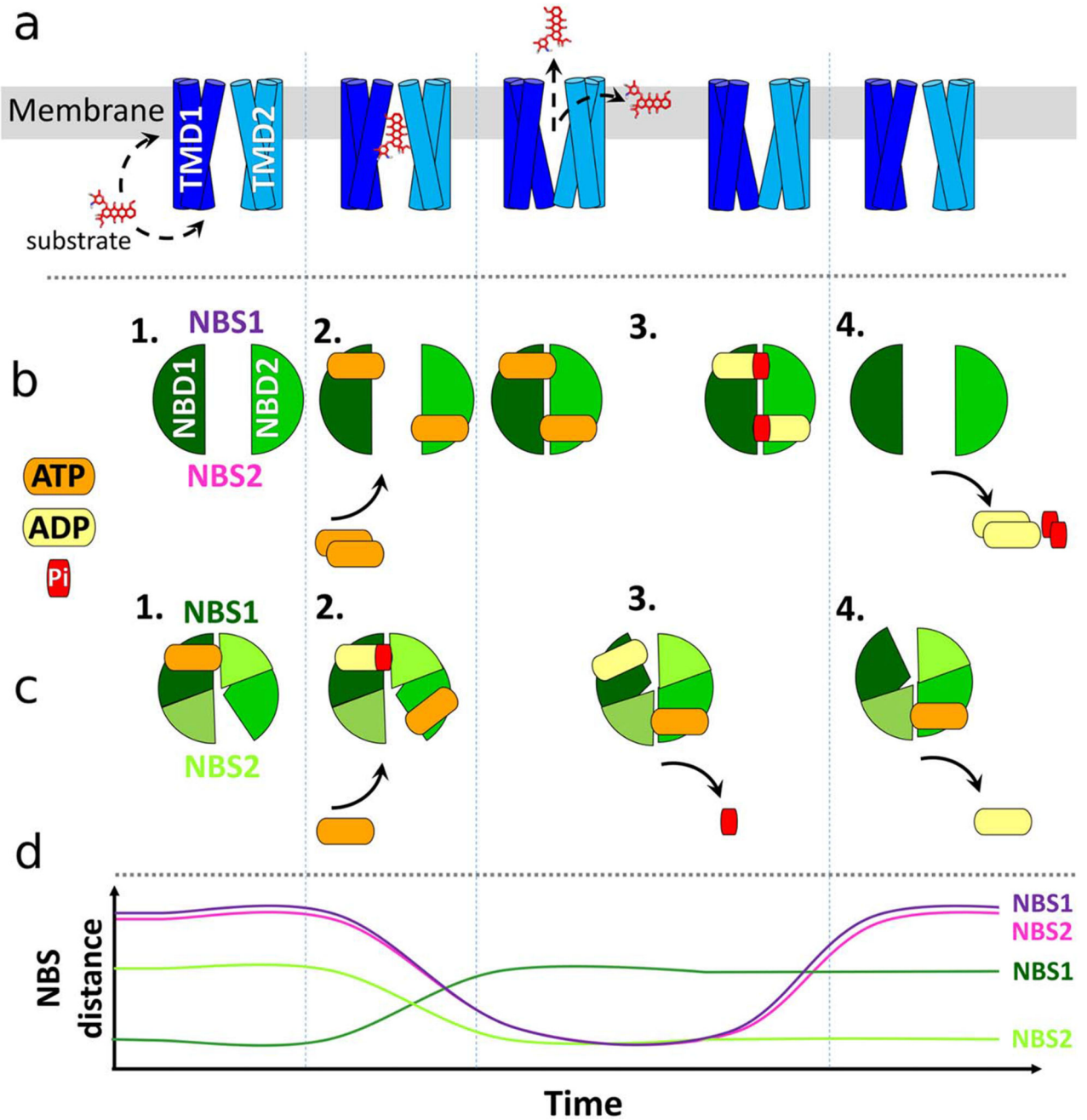


Fig. 2. Cartoon summarizing the two main types of transport models (with/without constant NBD contact).

a) Motions of the TMDs during the transport cycle, including the two possible paths (between membrane sides or between membrane leaflets) of substrate binding and release. b) NBD and nucleotide motions including full NBD separation (Tweezers-like [94], ATP switch model [95] and processive clamp [96–98]). c) NBD motions predicted for models assuming constant contact (alternating sites [99], constant contact [100] or nucleotide occlusion model [101,102]). d) Schematic illustration of the change in distances across the ATP binding site (from Walker A to ABC signature motif as a function of the transport cycle

(purple hues: NBS distances in NBD full-separation models; green hues: NBS distances in NBD constant contact models).

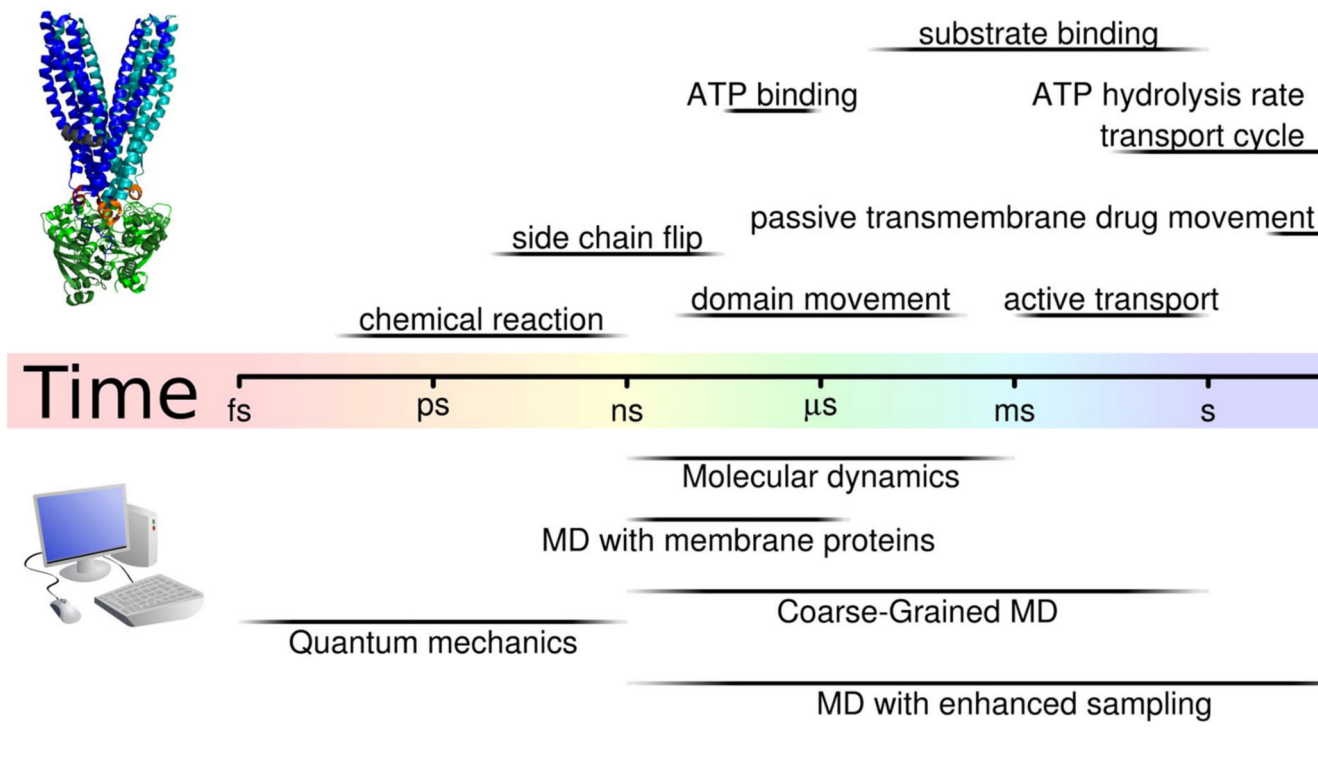


Fig. 3. Comparison of time scales: The top rows indicate the time scales of different biochemical processes, including events that are relevant for the transport cycle of ABC transporters. The bottom rows show times scales that are currently accessible to *in silico* techniques.

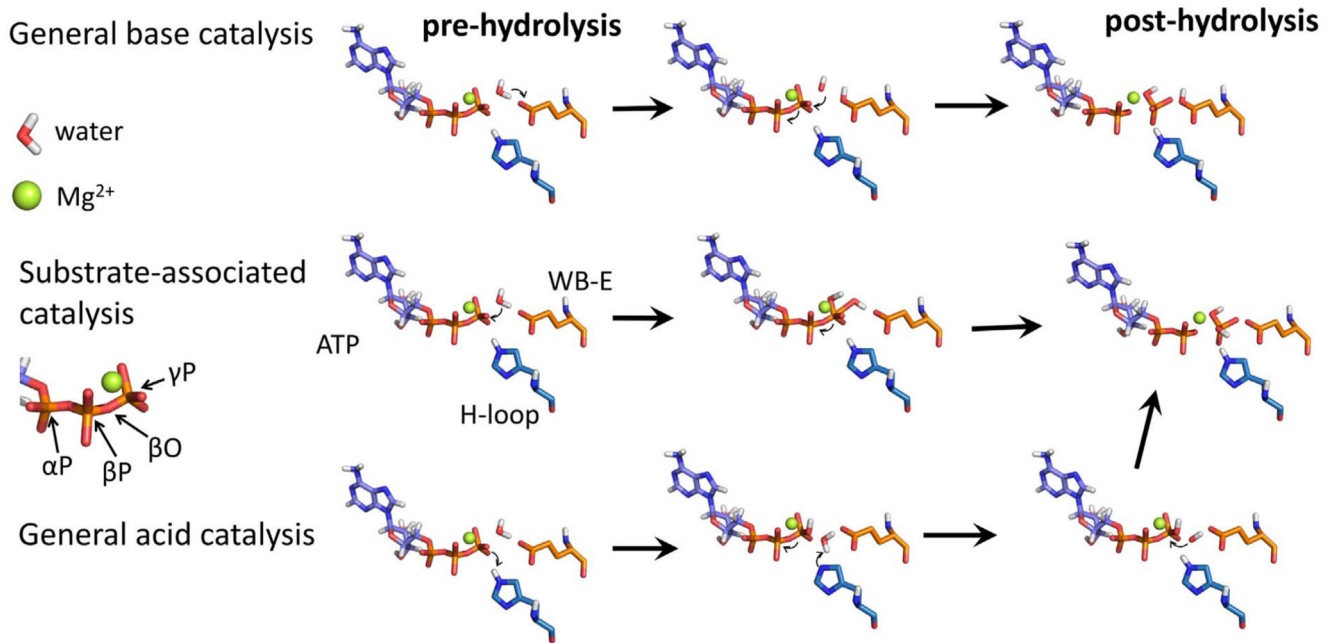


Fig. 4. ATP hydrolysis mechanisms showing the essential steps of the catalysis reaction of: top) the “general base catalysis”, middle) the “Substrate-associated catalysis” and bottom) the “general acid catalysis” reaction.

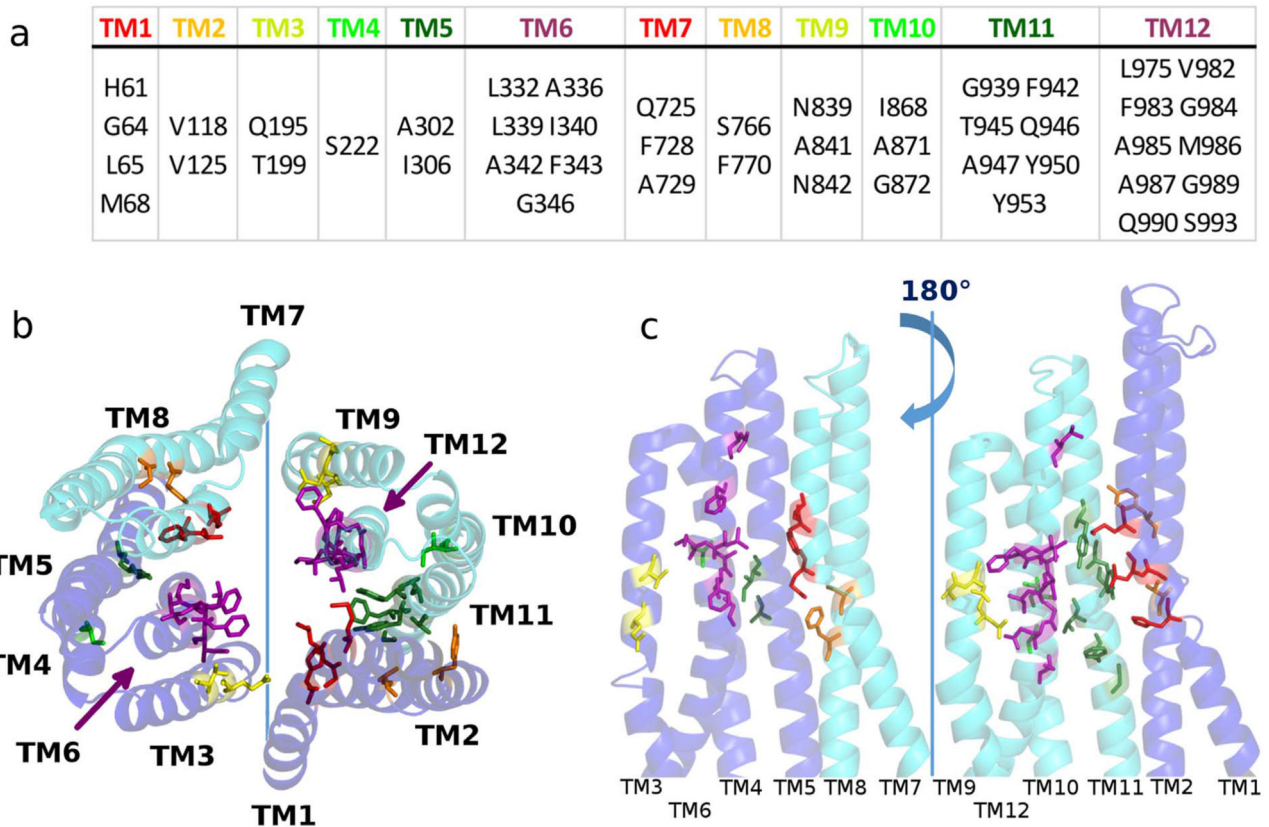


Fig. 5. Substrate interacting residues in ABCB1 [167,169,174].

a) Table lists interacting residues grouped by transmembrane helices. b) These interacting residues are highlighted on an outward facing model of ABCB1 viewed from the extracellular space. The model was built with modeller [178] using Sav1866 (PDB ID: 2HYD) [9] as template (c) Open book representation of TMD1 and TMD2 viewed from the substrate binding pocket (as indicated by the blue line in panel b).

Table 1
Consensus sequence and function of conserved motifs in the NBDs listed according to their occurrence from N- to C-terminus.

Motif	Consensus sequence	Function
A-loop [13]	(F/K)xY ^a	ATP binding (base stacking)
Walker A or P-loop [14]	GxxGxGK(S/T) ^a	ATP binding (phosphate binding)
Q-loop [15]	hV(S/P)Q ^b	TMD-NBD communication
X-loop [16,17]	TRVGDKGTQ	TMD-NBD communication
signature motif or C-loop [18]	LSGGQ(K/R)Q	Phosphate binding, NBD-NBD communication
Walker B [19]	hhhhDE ^b	ATP hydrolysis
D-loop [20,21]	SALD	NBD-NBD communication, unidirectionality of transport
H-loop [22]	hAHL ^b	ATP hydrolysis

^a x = any residue.

^b h = hydrophobic residue.

Table 2
Different catalytic models summarized briefly in four steps. (The Tweezer model that has been proposed based on the maltose importer is included for further reference and comparison).

Steps	Nucleotide occlusion [101–103]	ATP switch [95]	Processive clamp [96]	Alternating sites [99]	Constant contact [100]	Tweezers-like (in MalFGK2)[94]
1	NBDs: semi-open dimer NBS1: occluded ATP NBS2: loosely ATP binding TMDs: inward open	NBDs: separated, empty	NBDs: ATP binding, dimer formation	NBDs: semi-open dimer NBS1: ATP binding NBS2: empty TMDs: inward open, bound substrate	NBDs: semi-open dimer NBS1: occluded, ATP-bound NBS2: occluded, empty	NBDs: semi-open dimer NBSs: empty TMDs: inward open
2	TMDs: substrate binding	NBDs: ATP binding, dimer	NBS1 ^a : hydrolysis, maybe release of P _i	NBS2: ATP binding NBS1: hydrolysis → high energy ADP + P _i state TMDs: inward open	NBS1: occluded, hydrolysis open NBS2: TMDs: outward open, substrate release	Maltose loaded MBP binds to protein NBSs: ATP binding TMDs: outward open
3	NBS1: hydrolysis NBS2: occluded TMDs: outward open → substrate release	NBDs: hydrolysis	NBS2 ^a : ATP hydrolysis, release of P _i	NBS1: P _i release	NBS1: P _i release, open NBS2: binding ATP	NBDs: dimerize MBP: releases maltose
4	NBS1: ADP, P _i release TMDs:	NBD: ADP, P _i release, separated TMDs:	NBD: separated, ADP release	NBS1: ADP release TMDs:	NBS1: ADP release, empty, occluded NBS2: ATP, occluded	NBSs: ATP hydrolysis NBD: separated TMDs: inward open

Steps	Nucleotide occlusion [101–103]	ATP switch [95]	Processive clamp [96]	Alternating sites [99]	Constant contact [100]	Tweezers-like (in MalFGK2)[94]
	inward open	inward open		inward open → substrate release		release of maltose and MBP

MBP: Maltose-binding protein.

P_i: Inorganic phosphate or HPO₄²⁻.

^aSequential hydrolysis in NBS1 and NBS2 with unknown order.