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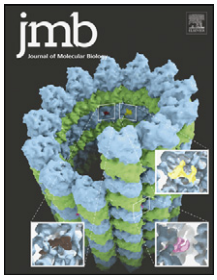

Mechanism of Action of ABC Importers: Conservation, Divergence, and Physiological Adaptations

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Abstract

The past decade has seen a remarkable surge in structural characterization of ATP binding cassette (ABC) transporters, which have spurred a more focused functional analysis of these elaborate molecular machines. As a result, it has become increasingly apparent that there is a substantial degree of mechanistic variation between ABC transporters that function as importers, which correlates with their physiological roles. Here, we summarize recent advances in ABC importers' structure–function studies and provide an explanation as to the origin of the different mechanisms of action.

The Different Classes of ABC Transporters

ATP binding cassette (ABC) transporters comprise a large superfamily of membrane proteins. In bacteria, fungus, plants, and animals, they transport molecules through the permeability barriers of cell membranes [1–4]. Cargo molecules are extremely diverse, including ions, small-to-medium-sized molecules such as sugars, amino acids, lipids, ionic metals, and large, bulky compounds including peptides, proteins, organo-metal complexes, and antibiotics. They are involved in many important physiological processes, such as plant development and growth, nutrient import, cellular detoxification, lipid homeostasis, signal transduction, antiviral defense, and antigen presentation [3,5–15]. From a clinical perspective, ABC transporters are of great interest as they are directly involved in tumor resistance to chemotherapeutics [16–18], drug resistance of parasites [19–21], fungal drug resistance [22], bacterial multidrug resistance, and bacterial virulence and pathogenesis [23–25]. All ABC transporters share a basic architecture comprising at least two intracellular nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs). The NBDs supply the energy via ATP binding and hydrolysis, while the TMDs form the transmembrane permeation pathway. An ABC transporter may function either as an importer or an exporter with the exporters present in all kingdoms and the importers present solely in bacteria and plants [26,27]. In bacteria, ABC importers require an additional protein partner: a substrate-binding protein (SBP) [28–32] that binds the substrate and delivers it to the TMDs. For many years, this large family of proteins was considered both mechanistically and structurally uniform yet, as more systems are characterized, cracks are beginning to appear is this assumption. For example, four significantly different 3D folds have been observed (Fig. 1), yielding the Type I and Type II exporter and the Type I and Type II importer classifications [33–36].

More recently, an additional structurally divergent group has been identified, the Energy-Coupling Factor transporters [37–44], which are referred to as Type III ABC importers. The structure and function of ECF transporters has been reviewed elsewhere [45–48] and will not be discussed here. The structural divergence of ABC transporters is accompanied by significant mechanistic variation, and several alternative mechanistic models have recently been suggested [33,49–52].

Herein, we focus on Type I and Type II ABC importers, present our perspective of their mechanistic conservation and diversification, and suggest a

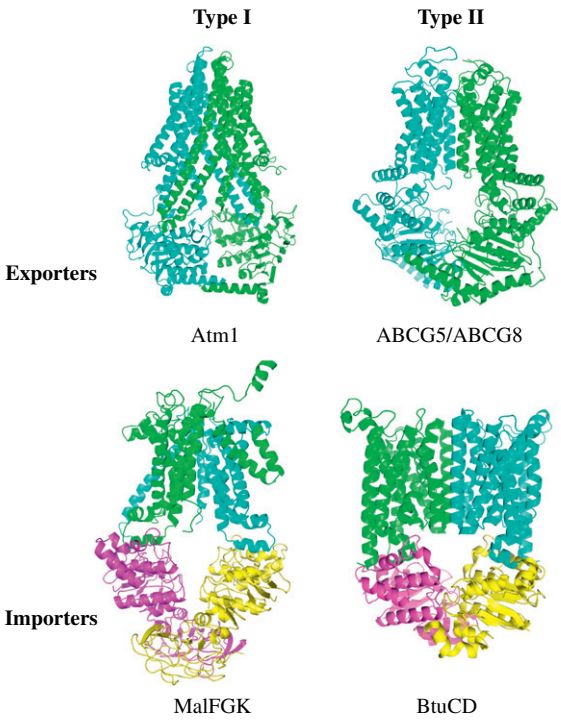

Fig. 1. The different folds of ABC transporters. Shown is the cartoon representation of the Type I metal-glutathione exporter Atm1 (PDB ID: 4MRV), the Type II sterol exporter (PDB ID: 5DO7), the Type I maltose importer MalFGK (PDB ID: 3FH6), and the Type II vitamin B_{12} importer BtuCD (PDB ID: 1L7V).

"biological logic" that explains the observed mechanistic speciation.

Common Ground: The NBDs and Mechanism of ATP Hydrolysis

All ABC proteins use the same motor to provide the driving force for the task they perform. Regardless of their physiological role, the 3D organization of this motor domain, or NBD, is highly conserved. For example, the RNase-L inhibitor is an ABC protein involved in ribosome biogenesis, formation of translation preinitiation complexes ,and assembly of HIV capsids, yet the structure of its NBD is extremely similar (RMSD 1.3 Å over 708 Cα atoms) to that of the vitamin B_{12} importer BtuCD (Fig. 2). The structural organization of the NBDs and the ATP binding sites has been extensively reviewed [34,50,53]. Briefly, two ATP binding sites are formed at the dimer interface between two NBDs, where residues from each monomer contribute to ATP binding and hydrolysis. The residues participating in ATP coordination and catalysis are highly conserved and are located in canonical motifs, including the Walker A/B and signature motifs and the Q, H, and D loops that define the superfamily of ABC proteins. These conserved motifs come together in

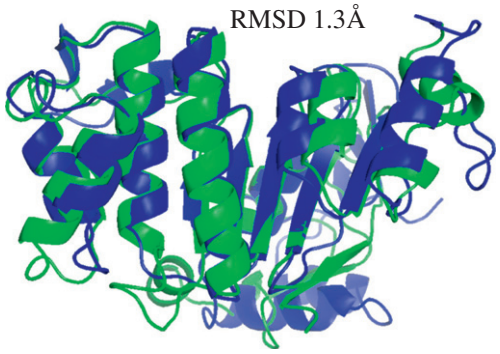

Fig. 2. Structural alignment between nucleotide-binding domains (NBDs) of two functionally unrelated ABC proteins. A single NBD from RNase-L inhibitor (blue; PDB ID: 1YQT) was aligned with a BtuD monomer (green; PDB ID: 1L7V), resulting in an RMSD value of 1.3 Å (over 708 Cα atoms).

the folded NBDs to form the ATP binding sites. The importance, and notably, the specific roles of these residues/motifs in binding and hydrolysis of ATP and to power transmission, appears to be highly conserved. One noteworthy example is the glutamate of the Walker B motif that serves as the catalytic base for ATP hydrolysis. The essential role of this glutamate has been demonstrated for prokaryotic and eukaryotic ABC transporters and for exporters and importers alike [54–61]. Other examples include the aspartate of the Walker B motif, the histidine in the H-loop, and the glutamine of the Q loop [59,62,63].

To hydrolyze ATP, the dimeric NBD's must come together to form a tight head-to-tail dimer sandwich [55], and this conformational change depends on ATP binding. This relationship between binding of ATP, the closure of the NBD dimer interface, and ATP hydrolysis is another feature that is conserved in all ABC proteins. It is often overlooked that ABC transporters can bind, hydrolyze, and drive transport by using nucleotides other than ATP. Several studies have shown that the affinities to CTP, GTP, and UTP are quite similar to those for ATP [64,65], which may be of importance, considering the high intracellular concentrations of all nucleotides [66,67].

First Signs of Divergence: Affinity, Kinetics, and Cooperativity of ATP Hydrolysis

The affinity (K_D, dissociation constant) of ABC transporters to ATP is rarely measured directly and is mostly inferred by the kinetic determination of the K_m of ATP hydrolysis. The K_m values differ over 3 orders of magnitude among the characterized ABC transporters, from the micromolar (e.g., BtuCD, MalFGK) to millimolar (e.g., Sav1866, HisPQM, Pgp) range [65,68–70], which was unexpected given the high 3D uniformity

and conservation of the catalytic residues. In prokaryotes, estimations of cellular ATP vary between 1 and 10 mM [66,71] while in animal and plant cells concentrations, as low as 0.1 mM has been reported [72–74]. Since K_m values of ATP hydrolysis are determined *in vitro*, their *in vivo* interpretation must be made with reservation. Keeping this in mind, the large variability in $K_{m(ATP)}$ may suggest that *in vivo*, some ABC transporters are constantly saturated with ATP, while others will run at full capacity only in certain cell types and/or under conditions of metabolic/energetic abundance. This phenomenon and its physiological importance and consequences remain unexplored. Unlike the variability of ATP binding affinities, the maximum turnover number, that is, the number of ATP molecules hydrolyzed per enzyme per unit time, (k_{cat}), is quite uniform and, in most cases, fall within the range of 0.6–2 s^{-1} [57,65,68–70]. There is no fundamental mechanistic requirement that ATP must be hydrolyzed at both sites for ABC transporters to function. Compelling evidence of this has been provided by a class of heterodimeric ABC exporters bearing a degenerate NBD unable to hydrolyze ATP (e.g., Ref. [59]). In these exporters, ATP is hydrolyzed only in one NBD, and this is sufficient to drive transport. In ABC transporters where both NBDs hydrolyze ATP, hydrolysis is often cooperative, with Hill coefficients approaching 2 [56,65,75].

In BtuCD and MalFGK, inactivation of one of the NBDs leads to an almost complete loss of ATPase and transport activity [56,76] while in HisPQM, 50% activity is retained [77]. This is surprising since MalFGK and HisPQM are quite similar, both being Type I importers and sharing a similar 3D fold, with a similar mechanism of transport. In almost every aspect, BtuCD is different from both MalFGK and HisPQM: it is a Type II importer, with a very distinct structure and transport mechanism. This uncharacteristic and mechanistic similarity between BtuCD and MalFGK, and the disparity between HisPQM and MalFGK, highlights our limited understanding of the molecular basis of allostery and cooperativity.

Mechanistic Differences between Type I and Type II ABC Transporters

Type I systems primarily import metabolites such as sugars, amino acids, peptides, and compatible solutes [78–81], while Type II systems import trace non-metabolites such as iron–siderophore complexes, hemin, and vitamin B_{12} [82–84]. It is generally accepted that the abundance of the substrates is reflected in the affinities of the two subgroups, and therefore, the binding/transport affinities of Type II systems are higher than those of Type I systems [50]. Indeed, Type II systems have been reported to bind their substrates with very high affinities with K_D values in sub-nanomolar to nanomolar range [83–85]. There is a considerable degree of variation in the binding affinities of Type I systems, from tens of nanomolars to several micromolars [86–90]. This variability is perhaps related to the coordinated function of some of these systems as complimentary high- and low-affinity import systems [91,92].

The structural differences between Type I and Type II transporters have been extensively reviewed elsewhere [33,36,53,93]; thus, for the purpose of this discussion, it will suffice to acknowledge that although these two groups share the same NBD fold, their TMDs are entirely different.

Hereafter, we examine each step of the transport cycle, highlighting the mechanistic differences between Type I and Type II ABC importers.

Step I, substrate recognition

Substrate binding is the first mandatory step in the transport process. For this, both Type I and Type II systems depend on their cognate SBPs [94,95]. In Gram-negative bacteria, the SBPs are believed to diffuse in the periplasm, although direct evidence for this is still lacking. In Gram-positive bacteria, the SBPs are fused to the membrane via a lipid anchor [94,96] or are directly fused to the TMDs. In the latter case, one, two, or even three SBPs may be found fused in tandem to homodimeric TMDs, and as such, the assembled transporters will have two, four, or six SBPs [32,96].

Already, at the stage of substrate recognition, differences between Type I and Type II systems are apparent. In all SBPs, the substrate binding pocket is formed between the N′ and C′ terminal lobes of the protein, which are connected by flexible loops in Type I systems and by a rigid α-helix in Type II systems [28,84,94,97–99]. The connecting loops of Type I SBPs enable the free movement of the lobes relative to one another, and upon substrate binding, the lobes close in a Venus flytrap fashion. Consequently, the apo and holo conformations of SBPs of Type I systems are significantly different (Fig. 3A) [98,100–103]. In comparison, the rigid α-helix in Type II systems bars the relative movements of the lobes, resulting in similarity between the apo and holo conformations of Type II system SBPs (Fig. 3B) [28,97]. This difference between Type I and Type II systems likely explains why Type II systems are insensitive to the presence of substrate: the TMDs of BtuCD and MolBC will respond similarly to the docking of their cognate SBPs, whether substrate is bound or not, and the ATPase activity of BtuCD and MolBC is equally stimulated by the SBPs, regardless of the presence of substrate (our unpublished results and Refs. [68,88]). Conversely, MalFGK and OpuA clearly differentiate between the holo and apo conformations of their SBPs, as only the holo form will lead to the closure of the NBD dimer and to the stimulation of ATP hydrolysis [69,104].

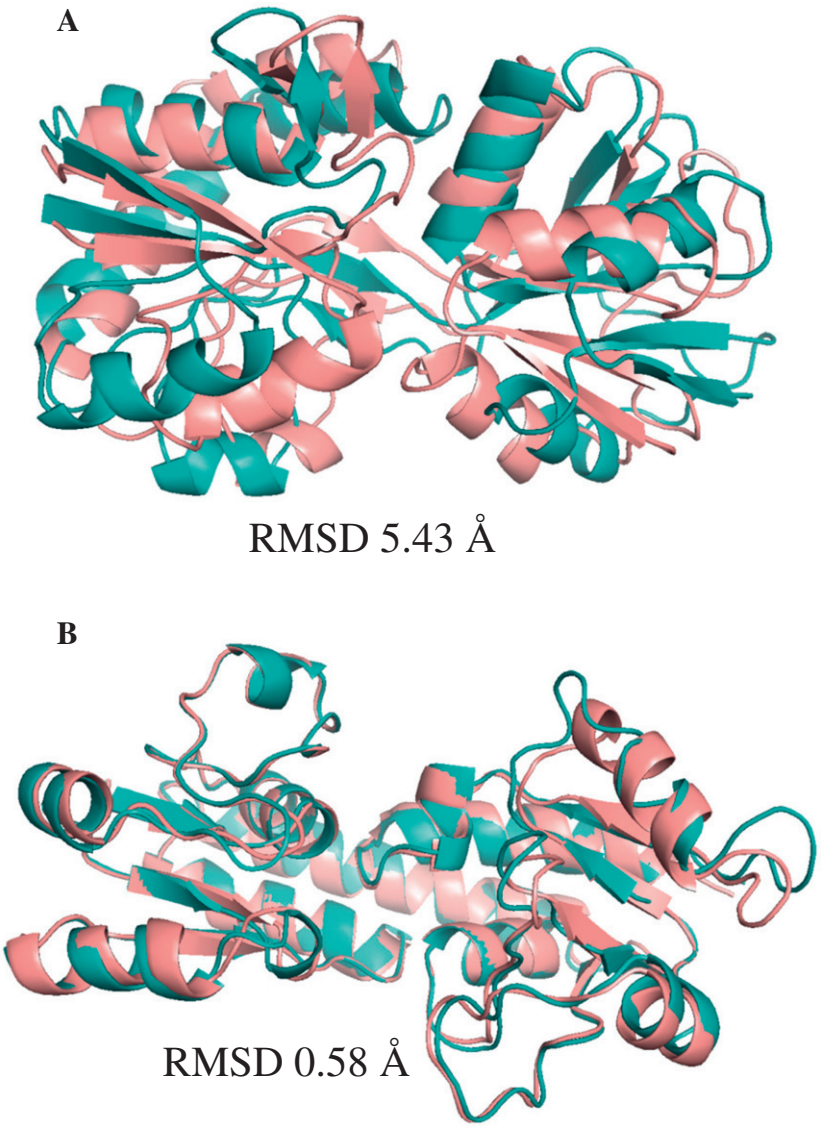

Fig. 3. Conformational changes in the SBPs of Type I and Type II systems upon substrate binding. (A) A bottom view of the structural alignment of apo (salmon pink) and holo (teal blue) glutamine SBP (PDB IDs: 1GGG and 1WDN, respectively), showing the lobes' closure upon substrate binding. The large conformational change that occurs upon substrate binding is mirrored in the RMSD (5.43 Å over 1688 Cα atoms). (B) Same as (A), only shown are the apo (salmon pink) and holo (teal blue) forms of vitamin B_{12} SBP, BtuF (PDB IDs: 1N4D, and 1N4A, respectively). Only a minor conformational occurs upon substrate binding (RMSD 0.58 Å over 1448 Cα atoms).

Step II, docking of the SBP to the TMD. What is the resting conformation?

Once substrate is bound to the SBP, it must be delivered to the transporter. For Type I systems, it is quite clear that in the resting state, the NBDs are separated from one another but are likely already ATP-bound, and the TMDs of the transporter are oriented toward the cytoplasm. Namely, in the resting state, the docking site for the SBP is not yet formed. During the initial interaction between the substrate-loaded SBP and the TMDs, the TMDs are reoriented to an outward-facing conformation that accommodates SBP docking, with the two lobes of the SBP open to discharge their cargo to the TMDs and the NBDs dimer close. This series of events, which in reality is one single concerted event, is the heart of the mechanism of action of Type I systems and accounts for the coupling between substrate presence, ATP hydrolysis, and transport [65,69,104]. In contrast, all Type II systems studied to date exhibit a considerable degree of basal ATP hydrolysis activity in the absence of the SBP and/or substrate. This uncoupled and wasteful activity has been observed across phyla and in both ABC exporters and Type II importers [59,68,88,105]. As the intracellular concentration of ATP (and other hydrolysable nucleotides) is very high (millimolar range), it is unclear how to define the *in vivo* resting state of Type II systems—if such a state exists. Unlike Type I systems, the docking site for the SBP is preformed in Type II systems, and its formation is independent of ATP binding and of a concerted conformational change. Relatively, there is little structural rearrangement in the TMDs that comes in

contact with BtuF; nothing that resembles the prominent, global, and conformational changes that occur in MalFGK upon docking of MalE [106,107]. Preformation of the SBP docking site is likely correlated with the different transporter–SBP interaction affinities of Type I and II systems. In Type I systems, the interaction between the SBP and the transporter is transient and of low affinity. This has been measured for the Mal [108], His [109], Met [110], Opp [111], and Mod [88]. The opposite is true for the Type II systems FhuBC-D, BtuCD-F, HmuUV-T, and MolBC-A that spontaneously form high-affinity (nanomolar to picomolar) and highly stable complexes with their respective SBPs [57,88,110,112]. Another clear difference between Type I and II systems lies in the role of ATP binding with respect to SBP docking to the transporter. In Type I systems, the transporter–SBP interaction affinity is lowest when the transporter is nucleotide-free, and ATP binding is essential to stabilize the interaction for a sufficient time to allow the SBP to deliver its cargo [88,107,113]. The exact opposite is true in Type II systems, where, in all studied cases, the highest interaction affinity is between the SBP and the nucleotide-free transporter, while ATP binding serves to displace the SBP [88,110,114].

Step III, the occluded state

Once the SBP docks to its cognate Type I transporter, the NBDs close to form a tight head-to-tail dimer [55]. This closure, which is required for ATP hydrolysis, only takes place if substrate is delivered, and this is the molecular mechanism that underlies coupling in Type I systems. No such coupling exists in Type II systems: the binding of ATP is sufficient to induce the closure of the NBDs (that leads to hydrolysis), independent of SBP and/or substrate, and this explains the high basal ATPase activity of Type II systems. None of the Type I and II systems studied to date appears to have a high-affinity membranous binding site for the substrate [110,115]. To facilitate substrate release from the relatively high-affinity binding pocket of the SBP, loops from the TMDs protrude into the SBP, distorting the binding pocket and effectively abolishing it [106,116]. The substrate is then transferred to an occluded cavity that is formed between the SBP and the permeation pathway, which is lined by the TMDs. Such an occluded state, as predicted originally by Jardetzky in 1966 [117], is a prerequisite of active transport and is common to ABC transporters, P-type ATPases, and secondary transporters [117–124]. In Type I transporters, this occluded state is extremely short-lived and is considered to be the high-energy catalytic intermediate of the transport cycle [119]. Since the NBDs have closed at this stage, ATP can be hydrolyzed, the NBDs separate, and the TMDs reorient to an inward-facing conformation. Once the reorientation of the TMDs has taken place, substrate is released from its low-affinity occlusion site to the cytoplasm. The analogous sequence of events in Type II remains somewhat unclear. Unlike maltose in the MalFGK-E complex, vitamin B_{12} was missing from the structure of the BtuCD-F complex, although holo-BtuF was used in crystallization [106]. Using several methods, we demonstrated that upon complex formation, vitamin B_{12} is spontaneously released from BtuF [110]. Whether this is an artifact of the detergent environment remains an issue of some debate [125]. Therefore, the point at which the substrate is released to the cytoplasm remains to be definitively determined.

Step IV, back to square one: resetting the system

The high-energy and occluded state of Type I systems is highly unstable, and once ATP is hydrolyzed, the SBP dissociates, showing a very fast k_{off} [88,110], the release of ADP follows, and the system is then free to rebind ATP to prepare for the next transport cycle. Contrarily, in Type II systems, once the substrate is released, the substrate-free transporter–SBP complex collapses into an asymmetric state of extreme, virtually unbreakable stability, exhibiting the lowest energy state of the transport cycle [106,110]. ATP binding and hydrolysis provide the energy necessary to break the complex and reset the system. We postulate that several rounds of ATP hydrolysis are required to release the SBP and could explain the poor ATP/vitamin B_{12} transport stoichiometry of BtuCD-F [56,68]. Curiously, in Type II systems, we and others have observed that high substrate concentrations facilitate SBP release. This has been reported for the Btu [110,126], Mol [88], and Hmu systems (H.K, O.L, unpublished results). The molecular basis of this phenomenon is poorly understood since it is believed that the substrate cannot access its SBP binding pocket while the latter is docked to the transporter.

Additional Points of Divergence: Conformational Changes and Allosteric Connectivity

Expertise in structural biology is not required to appreciate the extent of conformational changes that occur in MalFGK during its catalytic cycle. Even the untrained eye can easily detect the large bodies of mass that change their position in the shift from the outward- to inward-facing conformation. This is not the case for BtuCD where the structures of the different catalytic states show subtler difference, which are often local. Even the NBDs of Type II systems seem to open and close to a much lower degree than those of Type I systems and exporters. It is not only the extent of the conformational changes that is different, it is also their nature. In Type I systems, reorientation of

the TMDs from the inward- to the outward-facing conformation involves large-scale rigid body conformational changes, entirely consistent with an "alternating access" mechanism [34,115,127]. This is not the case in Type II systems, where alternating access (and occlusion) is achieved via orchestrated local movements of residues that serve as gates, which are somewhat similar to gate opening in ion channels. The sequential opening of the gates, and closing of the ones the substrate has already traversed, creates a "squeezing" effect that has been compared to peristalsis [120].

Long-distance allosteric connectivity is fundamental to the function of all ABC transporters, importers, and exporters alike. The opening and closing of the NBDs are transmitted to the TMDs, which in turn change their orientation. Similarly, SBP docking to the periplasmic side of the TMDs facilitates NBD closure. Although allostery is apparent in all studied systems, the connectivity and its output are very different. As mentioned, Type I systems are highly coupled, meaning that they do not hydrolyze ATP in the absence of substrate. This coupling is achieved through the strict allosteric connectivity between the periplasmic side of the TMDs and the NBDs, for example, the MalK dimer will only close upon the docking of substrate-loaded MalE to the periplasmic side of MalF and MalG.

Thus, in Type I systems, there is tight conformational coupling between the TMDs and NBDs; if the NBDs move, the TMDs will as well. This is not true for Type II systems. Figure 4 compares the structural rearrangements that take place in the NBDs and TMDs of MalFGK and BtuCD upon the docking of the cognate SBPs. When BtuF docks to BtuCD, each of the TM helices change their position (Fig. 4A, left panel) and the RMSD for the TMDs (before/after docking of BtuF) is 2.28 Å over 4251 Cα atoms. Unlike its effect on the TMDs, BtuF docking has little effect on the conformation of the NBDs (Fig. 4A, right panel) with an RMSD of 0.52 Å (over 2968 Cα atoms), demonstrating the conformational decoupling between the TMDs and the NBDs. Similar conclusions are drawn from DEER and EPR experiments [126,128], which showed that upon the addition of ATP (in the absence of BtuF), the NBDs close but the periplasmic gates of the TMDs remain irresponsive. Such conformational decoupling is not observed in Type I systems. Upon MalE docking to MalFGK, the TMDs shift with an RMSD of 2.83 Å (Fig. 4B, left panel), accompanied by a comparable shift in the NBDs with an RMSD of 2.55 Å (over 3812 and 5564 Cα atoms, respectively; Fig. 4B, right panel). The conformational decoupling observed in Type II systems leads to the inevitable conclusion that at least two parallel catalytic cycles of ATP hydrolysis are possible in these systems. In the uncoupled cycle (absence of SBP/substrate), the NBDs open and close; ATP is thus hydrolyzed, but the gates of the TMDs remain indifferent to this activity. In the full-fledged catalytic cycle (presence of SBP/substrate), the NBDs open and close in the same manner, but the gates of the TMDs respond accordingly.

Non-Stochastic Mechanics

In the absence of its substrate, the vitamin B_{12} ABC importer BtuCD has a very high level of basal ATPase activity [56,68,110,129]. Such uncoupled ATP hydrolysis is also a feature of at least one other Type II ABC importer [88] and of ABC exporters [59,105]. It is unknown whether this phenomenon also occurs *in vivo*, but high levels of uncoupled ATP hydrolysis have been observed independently using a variety of *in vitro* approaches for prokaryotic, yeast, and mammalian systems [59,68,88,130]. For BtuCD, this has been observed in detergent solution [56,68,110], nanodisks [129], liposomes [56,68], and in membrane fractions (O.L., unpublished results). We therefore currently hypothesize that such is also the case *in vivo*.

If indeed this is the case, this means that BtuCD and perhaps other ABC transporters are constantly hydrolyzing ATP, that is, in a state of constant activity. Such constant activity, especially if continued, is routinely observed to adversely affect the stability and performance of man-made machines. Intuitively, one may expect that an enzyme, which consumes energy and has moving parts, will also be negatively affected by prolonged work. We therefore decided to test what happens to BtuCD as it hydrolyzes ATP for a long time. As summarized below, the results were very surprising [129].

We let BtuCD hydrolyze ATP in the uncoupled mode (absence of BtuF and vitamin B_{12}) for minutes, hours, or days, and we followed its stability and activity levels. The first thing we noted is that BtuCD is not adversely affected by prolonged work. On the contrary, it is protected by it. Idle BtuCD aggregated and lost activity much faster than ATP-hydrolyzing BtuCD. The relative stability of the "working" enzyme could be attributed to a stabilizing effect of the dwell times of BtuCD in the nucleotide-bound conformations. This means that the stabilizing effect of nucleotide binding, even if short-lived, outweighs any unfavorable effect imposed by the constant conformational changes, production of heat, and/or higher energy states sampled by ATP-hydrolyzing BtuCD. Although such stabilization clearly has evolutionary/biological advantageous, it is surprising with respect to the properties of the protein polymer.

More surprisingly, we observed that during the hours of continuous ATP hydrolysis, a larger fraction of the BtuCD molecules adopt a conformation to which BtuF successfully docks. This "trained" population of BtuCD molecules had the same ATPase activity (K_{cat}, K_m, n_{hill}) as the "original" one and also the same equilibrium and pre-equilibrium rate constants (K_D,

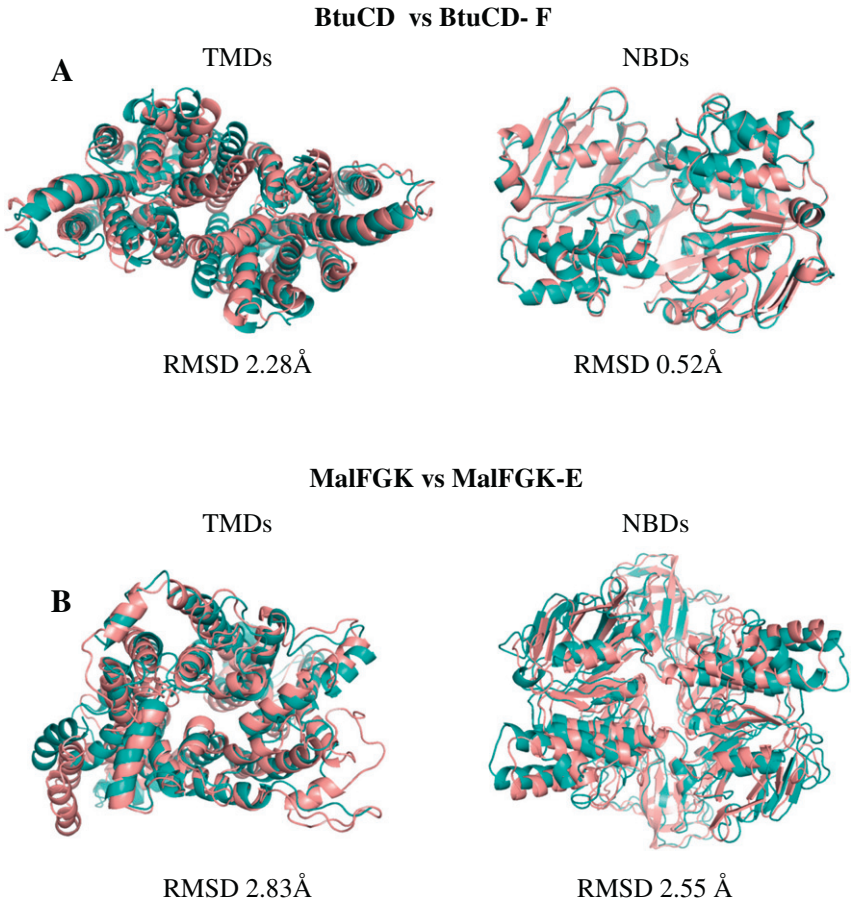

Fig. 4. The effect of the docking of the SBP on the conformational changes of Type I and Type II transporters. (A) The TMDs and NBDs of BtuCD (teal blue) and BtuCD-F (salmon pink;PDB IDs: 1L7V and 2QI9, respectively) were aligned separately to highlight the structural rearrangements of each domain. Molecules are shown from the top. (B) Same as (A), only for MalFGK (teal blue; PDB ID: 3FH6) and MalFGK-E (salmon pink; PDB ID: 3PV0). In BtuCD, the TMDs are affected by the docking of the SBP (RMSD 2.28 Å over 4251 Cα atoms), while the NBDs hardly are (RMSD 0.52 Å over 2968 Cα atoms), demonstrating the facultative decoupling between the NBDs and the TMDs. In MalFGK, both domains are equally affected (RMSD 2.83 Å *versus* 2.55 Å, over 3812 and 5564 Cα atoms, respectively), and their movement is obligatorily coupled.

k_{on}, k_{off}) for BtuF binding. The only parameter that changed was the maximal binding, B_{max} (or E_T in this case), for the BtuCD–BtuF interaction. Concomitant with its improved BtuF-binding capacity, the "trained" population of BtuCD molecules also displayed higher rates of vitamin B_{12} uptake in a liposome reconstituted systems [129]. Importantly, incubation of BtuCD in any of the states that mimic the intermediate states of ATP hydrolysis (ATP-bound, transition state, or ADP-bound) did not lead to this improved activity. In other words, to improve its performance, BtuCD must repeatedly go through the full catalytic cycle of ATP hydrolysis.

Traditionally, enzymes and proteins are viewed as being Markovian, that is, operating by a stochastic process, where the future is determined only by the present state and not by any past states. However, advances in single-molecule techniques have proven that this is not always true, as some enzymes clearly show "memory effects" [131–135]. For example, the speed of catalysis for a given catalytic cycle can be affected by the amount of time passed since the last cycle [131]. The molecular basis for this intriguing "conformational memory" is hysteresis [136–141], which occurs when there are at least two conformers differing in their activity levels and/or kinetic properties. When the conformational change between these conformers is slower than the enzymatic turnover rate, hysteresis is observed [142]. Changes in the activity level of the enzyme may be manifested by changes in K_{cat}, K_D, or E_T (or B_{max}), as is the case with BtuCD. Using spectroscopy, we indeed observe that BtuCD undergoes a very slow conformational change that takes place when the protein is constantly hydrolyzing ATP for several hours. As observed with the improved activity, incubation of BtuCD in any of the intermediate states of ATP hydrolysis did not lead to this slow conformational change.

Such slow conformational changes, which occur on a time-scale of hours, are rare but not unheard of [132,135].

Termination of ATP hydrolysis triggers the backward conformational change, that is, the return to the basal state. This reverse conformational change is also very slow, taking hours to complete. Consequently, BtuCD displays the so-called "conformational memory": it remains in the more active conformation for several hours after the termination of catalysis before slowly relaxing back to its basal state [129].

We do not know whether other ABC transporters also feature non-stochastic mechanics. For two other ABC transporters that we tested (MetIN and ModBC), we found no evidence for hysteresis, improved activity, or conformational memory. Unlike these two Type I importers, preliminary work with the BtuCD-like Type I importer MolBC-A [88] suggests that it also displays some non-stochastic characteristics (N.L.L, M.K., O.L, unpublished results).

The *in vivo* relevance of these observations is unclear. Yet, it is tempting to speculate that they provide a "biological logic" to the long-standing observations of the uncoupled ATPase activity of BtuCD and other ABC transporters [59,88,105].

The Black Box: Are there Other Types of ABC Transporters?

A phylogenetic analysis of ABC transporters revealed that the transporter superfamily can be divided to at least 14 different subgroups [7]. Structural classification of SBP-dependent importers identified seven subclasses [94,95]. We therefore suspect that the current division to Type I and Type II is probably as crude as distinguishing between bacteria using Gram staining. As more structural and functional details are revealed, we are bound to uncover differences between transporters that are considered similar today. Such speciation is already apparent among BtuCD, MolBC, and HmuUV—three Type II transporters that share very similar structures and mechanisms of action [57,143–146]. However, MolBC exhibits the highest uncoupled ATPase activity, which is completely irresponsive to the presence of its cognate SBP/substrate. BtuCD has an intermediate level of uncoupled ATPase activity and shows mild (twofold) acceleration of ATPase activity in the presence of BtuF and vitamin B_{12}. HmuUV shows the lowest level of uncoupled ATPase activity of the three but is the most responsive to HmuT and hemin (four- to fivefold stimulation). Intergroup speciation is also observed between Type I transporters, for example, MalFGK and HisPQM (e.g., compare Refs. [69,147] to [65,76] to [77]). Our prediction is that future research will demonstrate that rather than discrete mechanistically defined clusters, there exists a continuum of mechanistic speciation, where each transporter shows role-specific adaptations.

Physiological Relevance: Why Do Type I and Type II Systems Employ Such Different Mechanisms of Action?

Table 1 summarizes the central mechanistic differences between Type I and Type II systems, raising questions as to why such different modes of transport evolved. We propose that these differences are dictated by the nature of the substrate. Type II systems import compounds that are present in the environment at very low concentrations, whereas Type I system substrates are relatively abundant (sugars, amino acids). In addition, the physiological need for vitamin B_{12}, for example, cannot be satisfied by importing an alternative substrate. In contrast, most of the substrates of Type I systems are interchangeable. For example, maltose imported as an energy or a carbon source can be exchanged for other molecules (e.g., histidine). Moreover, the number of molecules required by the cell may also impact the need for coupling, for example, to form one *Escherichia coli* cell, only 10–20 molecules of vitamin B_{12} are needed [148] but millions or even billions of energy or carbon source molecules are required [149]. For this reason, Type I systems must be highly coupled, as it would be disastrous to expend 50–100 molecules of ATP for each imported molecule of maltose or glucose. No such evolutionary pressure limits the price tag for the import of scarce and non-interchangeable yet essential compounds.

In a sense, Type II systems operate by a "buy now, pay later" mechanism: the scarcity of the substrates demands that the SBPs of Type II systems be high-affinity scavengers. In these SBPs, the substrate binding pocket is almost preformed and no major structural rearrangements are required to trap the precious cargo. This ensures that the binding has a fast k_{on} and a low K_D (high affinity). Since K_D is directly proportional to the ΔG of binding, the substrate binding energy of SBPs of Type II systems will have a large negative value. Despite this, in Type II systems, the substrate-loaded SBP spontaneously binds to its transporter with extremely high affinity with no requirement for an energy input in the form of ATP binding or hydrolysis. This means that the ΔG of complex formation is also negative and that the system is in a very stable, low-energy state. At this point in the cycle, the substrate is already occluded or even transported [110,150] yet no "ATP price" was paid. Effectively, the cell made sure it got what it needed, but at this point, the system is "stuck" in its lowest energy pit, and the price, perhaps even an exaggerated one, embodied in the poor transport stoichiometry must be paid. In clear distinction, Type I systems cannot operate this way. An exaggerated price tag for maltose would be inexcusable and this is why the presence of substrate plays a

Table 1. Mechanistic similarities and differences between Type I and Type II systems

	Type I	Type II
Systems	MalFGK, HisPQM, GlnPQ, OpuA	BtuCD, MolBC, HmuUV, FhuBC
Transported substrates	Ions, sugars, amino acids, compatible solutes	Iron–siderophore complexes, vitamin B12, hemin
Affinity to substrate	Low to medium (low to high micromolar)	Nanomolar
Affinity to ATP	Varies, low to high micromolar	
V_{max} of ATPase	0.3–1 μmol mg^{-1} min^{-1}	
Cooperativity of the NBDs	Mostly cooperative, n_{hill} = 1.7-2	
Structural rearrangements of the SBP upon substrate binding	Prominent, Venus-trap like closure of the SBP around the substrate	Apo and holo forms of the SBP are very similar.
Basal ATPase rate	Low	High
Stimulation of ATPase rates by the SBP	High	Low
Affinity of the transporter–SBP interaction	varies, tens nanomolar to tens micromolar	Very high: Nano- to picomolar
Effect of ATP on the transporter–SBP interaction	ATP binding stabilizes the interaction.	ATP binding destablizes the interaction.
Effect of substrate on the transporter–SBP interaction	Substrate binding by the SBP promotes its association with the TMDs.	High substrate concentrations inhibit the SBP–transporter interaction.
Conformation of the ATP-bound transporter	Outward-facing (following docking of the SBP)	Inward-facing
Conformational coupling between the TMDs and the NBDs	Movements of the TMDs and the NBDs are tightly coupled.	In the absence of the SBP, the movements of the TMDs and the NBDs are uncoupled.

central role in initiating the activity of these systems. The SBP does not have a preformed binding pocket for the substrate; it must close around it to bind it. Similarly, there is no preformed docking site for the SBP on the transporter, and SBP docking necessitates a concerted structural change in both the SBP and the transporter. Cash is paid upfront: ATP binding and hydrolysis are required for the formation of the transport complex. At the same time, strict coupling between presence of substrate and closure of the NBDs ensures that a fair price is paid.

Future Directions: We Have Probably Just Scratched the Surface

Given the size of the superfamily and the involvement of ABC transporters in a wide spectrum of physiological pathways, it is likely that we have just begun to appreciate the extent of their mechanistic diversity. As noted above, physiologically related speciation has already been observed even though only a handful of systems have been well-characterized. For a better understanding of ABC transporters, we need to expand our studies to include more systems—especially ones from underrepresented groups. The importers of transition metals are one such group. Despite their broadly demonstrated importance to bacterial virulence and pathogenesis [24,151–155], nothing is known about the mechanism or structure of these transporters. Preliminary analyses have suggested that they have a smaller number of TM helices (relative to Type II systems) and that their mechanism of action may differ since they lack the determinants that mediate stable complex formation [57,152]. In addition, the *in vivo* context of ABC transporters, querying the number of transporter and SBP copies per cell and spatial transporter-SBP–outer membrane transporter segregation, also demand attention. Moreover, analysis of the interaction of ABC transporters with the ExbB/ExbD/TonB energy relay system and the determination of functional "rafts" formation remains to be addressed, potentially using super high-resolution microscopy, as has been applied to other fields in microbiology [156–158]. At the same time, application of advanced single-molecule techniques [90,159–163] will reveal mechanistic details that are lost by population averaging approaches and might disclose additional non-Markovian aspects of ABC transporters. Combining super high-resolution microscopy *in vivo* and advanced *in vitro* single-molecule approaches will take us to the next level of understanding the function and mechanism of these sophisticated molecular machines.

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Abbreviations used:
ABC, ATP binding cassette; NBD, nucleotide-binding domains; TMD, transmembrane domain; SBP, substrate-binding protein.

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