REVIEW ARTICLE

Diversity in ABC transporters: Type I, II and III importers

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Abstract

ATP-binding cassette transporters are multi-subunit membrane pumps that transport substrates across membranes. While significant in the transport process, transporter architecture exhibits a range of diversity that we are only beginning to recognize. This divergence may provide insight into the mechanisms of substrate transport and homeostasis. Until recently, ABC importers have been classified into two types, but with the emergence of energy-coupling factor (ECF) transporters there are potentially three types of ABC importers. In this review, we summarize an expansive body of research on the three types of importers with an emphasis on the basics that underlie ABC importers, such as structure, subunit composition and mechanism.

Introduction

ABC transporters, present in all organisms, represent one of the largest transmembrane spanning super-families. ABC stands for ATP-binding cassette due to the family's characteristic ability to bind and hydrolyze ATP in order to transport substrates across the lipid bilayer (Higgins, 1992). Found in both prokaryotes and eukaryotes, the well-studied ABC exporters, play a fundamental role in biological processes (Dean et al., 2001), are associated with multidrug resistance (Eckford & Sharom, 2009; Kerr et al., 2010; Seeger & van Veen, 2009), several human diseases (Dean et al., 2001; Fletcher et al., 2010) and have been extensively reviewed elsewhere (Al-Shawi, 2011). ABC importers play a major role in nutrient uptake, pathogenicity and virulence, making a focus on this diverse set of transporters ever more prevalent. ABC importers found in plants, prokaryotes and archaea are responsible for the transport of vital nutrients across lipid bilayers. In addition, many prokaryotic pathogens utilize ABC importers to circumvent the hosts innate immunes response, which makes this group of transporters of particular interest.

All ABC transporters have a common basic architecture with two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs). These domains assemble to form the basic unit of an ABC transporter. For ABC exporters, the TMD and NBD domains can be fused in multiple ways resulting in one-, two- or four-chain transporters, where the order of the domains may vary.

For importers, the core TMD and NDB subunits are individual chains, which can assemble to form homo- or hetero-dimeric TMDs bound to typically homodimeric NBDs. The NBD is classified by a series of motifs, including the Walker A (P-loop), Walker B and ABC signature motif, which are common in, but not limited to ABC transporters (Higgins et al., 1986; Walker et al., 1982). These motifs, in conjunction with additional conserved NBD regions (H-loop/switch region, Q-loop), play a role in ATP binding (Schneider & Hunke, 1998). The binding and hydrolysis of ATP in the NBDs are thought to control the opening and closing of the TMDs.

Unlike the NBD, the TMD sequence is considerably less conserved. For ABC importers, even the number of helices per TMD subunit can vary from 5 to 10, yielding a total of 10–20 transmembrane helices per transporter (Davidson et al., 2008). TMDs of the ABC uptake systems contain the so-called EAA motif (L-loop), a cytoplasmic loop containing two short helices that couple conformational shifts of the NBDs to the TMDs (Saurin & Dassa, 1994, Saurin et al., 1994). Together, the TMDs and NBDs form a complex to make up the core unit of an ABC transporter. For most ABC importers, substrate is delivered to a core importer complex through a substrate-binding protein (SBP). SBPs bind to a vast array of substrates with affinities in the nanomolar to micromolar range. These proteins are located in the periplasm of gram-negative organisms or tethered to the membrane in gram-positive bacteria. The most recent discovery of ECF or energy coupling factor transporters has revealed a divergence in ABC importers. ECF transporters contain both NBDs and TMDs but do not have an additional SBP for substrate delivery. Instead, a membrane-embedded component of the ECF transporter binds substrate. The overall topology and

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mechanisms each type of importer uses to transport substrates emphasizes the diversity amongst ABC importers.

Intact prokaryotic structures reveal diversity amongst ABC importers

Structures of ABC importers have revealed a divergence in overall architecture that is not quite understood. To date, 12 high-resolution crystal structures of importers have been solved, all of prokaryotic origin. Based on overall topology and mechanism of transport, several structurally characterized ABC importers fall within the Type I category including: multiple conformations of the maltose transporter $MalFGK_2$ (Chen *et al.*, 2013; Khare *et al.*, 2009; Oldham & Chen, 2011; Oldham *et al.*, 2007), methionine transporter MetNI (Kadaba *et al.*, 2008) and molybdate transporters ModBC from *Archaeoglobus fulgidus* (Hollenstein *et al.*, 2007) and *Methanosarcina acetivorans* (Gerber *et al.*, 2008). These differ from Type II importers which have been classified based on the structures of the vitamin B_{12} transporter BtuCD (Locher *et al.*, 2002), the heme transporter HmuUV (Woo *et al.*, 2012), and the molybdate transporter MolBC (HI1470/1) from *Haemophilus influenzae* (Pinkett *et al.*, 2007). The structural diversity of ECF transporters or Type III ABC importers was revealed with the full length structures of the folate (Xu *et al.*, 2013) and the hydroxymethyl pyrimidine (Wang *et al.*, 2013) transporters from *Lactobacillus brevis*.

Previous reviews have covered the conserved aspects of ABC transporters (Davidson *et al.*, 2008) or focused on structural characteristics (Biemans-Oldehinkel *et al.*, 2006) and the initial division of Type I and II importers (Locher, 2009; Oldham *et al.*, 2008). Since then much has been added to the field that expands our current understanding of transporter diversity, including ECF transporters, which have been the focus of several recent reviews (Erkens *et al.*, 2012; Slotboom, 2014; Zhang, 2013). This review is a culmination of the recent advances for all three types of ABC importers, with a focus on structures and mechanisms employed to transport substrates across the lipid bilayer.

Type I ABC importers

Structural and biochemical evidence has allowed for the division of the different types of ABC importer. In general, the Type I importers contain fewer transmembrane helices in comparison with Type II. In the absence of SBPs, Type I transporters show negligible levels of ATPase activity; however, activity is stimulated by ligand-loaded SBP (Bao & Duong, 2012; Davidson *et al.*, 1992). The structurally characterized Type I importers shed light on the diversity of transmembrane domain topology (Figure 1). The Type I importer MetNI has a minimal set of 5 TM helices from each TMD, which forms a homodimer totaling 10 helices (Kadaba *et al.*, 2008). The structures of two molybdate transporters (ModBC homologs) have an additional TM helix per monomer, totaling 12 helices (Gerber *et al.*, 2008; Hollenstein *et al.*, 2007). Unlike other ABC importers, which form homodimers, the TMDs of maltose transporter form a heterodimer of MalF and MalG, which have 8 and 6 helices, respectively. Despite their functional diversity, comparison of TMDs for the four Type I importers revealed that

the four helices that display ''up and down'' topology form a common arrangement of TM fold for Type I importers.

In addition, some Type I importers posses a regulatory domain (RD) attached to the highly conserved NBDs, which controls the function of these importers. Three of the four structurally characterized Type I importers (all except ModBC of *A. fulgidus* [AfModBC]; Hollenstein *et al.*, 2007) have RDs. The RDs of MalFGK (Oldham *et al.*, 2007) and ModBC of *M. acetivorans* [MaModBC] (Gerber *et al.*, 2008) have a similar overall fold displaying an elongated beta-barrel composed of two beta-sandwiches. The RD of MetNI exhibits a ferredoxin-like fold (Kadaba *et al.*, 2008). Once substrate or regulatory proteins bind to the RD, ATP hydrolysis and substrate transport are halted. By modulating transport activity, accessory domains such as the RDs can provide additional function to the core transporter.

Type I importers transport substrate by an alternating access mechanism

Although the number of TMD helices in Type I importers can vary, they transport their substrates by the same alternating access model (Jardetzky, 1966). Importers alternate between two conformations, inward- and outward-facing where the translocation pathway will be exposed to only one side of the membrane.

The detailed transport mechanism employed by Type I transporters can be explained by the maltose transporter, which is one of the best-characterized Type I systems. The transport of maltose is initiated by the binding of substrate-loaded MalE to nucleotide-free MalFGK (Oldham & Chen, 2011; Figure 2, State 1). Substrate-loaded MalE triggers a conformational change of MalFGK, inducing partial closure of the MalK dimer (Figure 2, State 2). When the NBDs are in the nucleotide-free semi-open state and substrate-loaded MalE is bound, the TMD subunits rotate toward the center of the transporter, narrowing the translocation pathway. However, the periplasmic side of the transporter still remains closed. The binding of two ATP molecules to MalFGK in the pre-translocation state induces conformational changes, including closure of the MalK dimer, opening of MalE, and a shift to the outward-facing conformation of the TMDs by a rigid-body rotation (Oldham *et al.*, 2007; Figure 2, State 3). EPR studies show that MalE alternates between an open and closed conformation depending on the binding of ATP to MalK (Austermuhle *et al.*, 2004). Taken together with the result that both MalE and ATP are required to close the MalK dimer (Orelle *et al.*, 2008), the EPR studies indicate that communication between MalE and MalK across the membrane is a major step of the transport process. By shifting from the pre-translocation state to the outward-facing state, MalFGK is able to transfer maltose from MalE to a temporary binding site in MalF (Oldham *et al.*, 2013; Figure 2, State 4). Upon hydrolysis of ATP, ADP and inorganic phosphate are released and MalFGK returns to the resting, inward-facing conformation. In this state, the closed MalK dimer is opened and MalE departs from the transporter.

Additional structures of Type I ABC importers have confirmed the mechanism defined by the maltose transporter. The structures of ModBC from *A. fulgidus* [AfModBC]
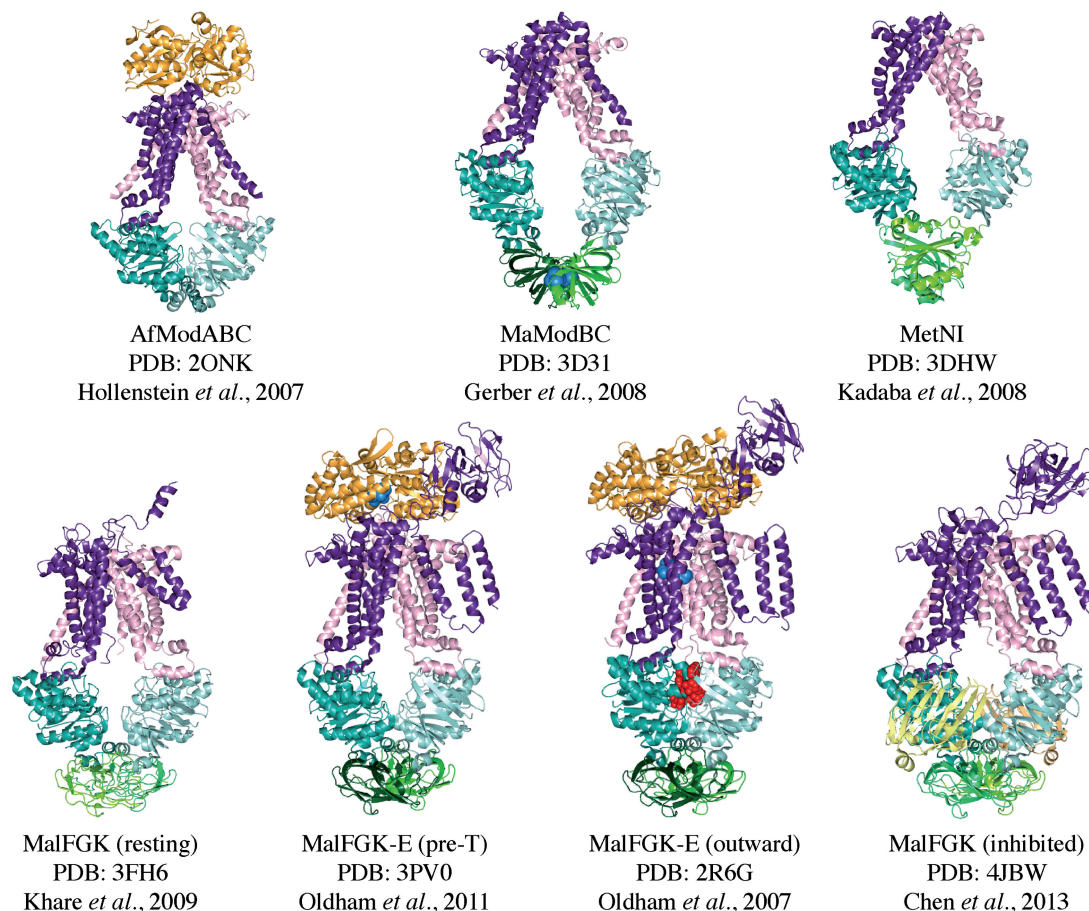
Figure 1. Crystal structures of Type I ABC importers. The TMD subunits are colored pink and deep purple, the NBD subunits are colored light cyan and teal, SBPs orange, RDs dark and light green, the regulation factor EIIA is pale yellow, the substrates are blue spheres and nucleotides are red spheres. From left to right on the top row, the following structures are represented in nucleotide-free conformations: ModBC of *A. fulgidus* [AfModB$_2$C$_2$-A], ModBC of *M. acetivorans* [MaModB$_2$C$_2$], and methionine transporter MetN$_2$I$_2$ from *E. coli*. MaModB$_2$C$_2$ is shown in the inhibited state with molybdate bound to the RDs. The bottom row shows structures of the maltose transporter from *E. coli*, MalFGK$_2$-E in the following conformations (from left to right): inward-facing; pre-translocation state; outward-facing, nucleotide bound; and inhibited, EIIA^{glu}-bound.

(Hollenstein *et al.*, 2007), and MetNI (Kadaba *et al.*, 2008) further confirm the conformation of the resting state for a Type I transporter; without nucleotide and in complex with substrate-loaded SBP. In the resting state, Type I importers adopt an inward-facing conformation, where the translocation pathway is open to the cytoplasm and the two NBDs adopt nucleotide-free, open conformation. Then, binding of ATP to the NBD dimer brings the NBD subunits together as observed in nucleotide-bound MalFGK (Oldham *et al.*, 2007) resulting in outward-facing conformation where the substrate-binding site will be exposed to the periplasm.

Due the presence of the regulatory domain, an additional conformation is allowed in Type I importers. The structure of MaModBC represents the trans-inhibited state, where two tungstate ions were bound to the regulatory domains of the ModC NBD dimer. (Figure 2, State 5; Gerber *et al.*, 2008). In this trans-inhibited state, MaModBC displays a larger distance between the coupling helices relative to other importers. The bound tungstate ions inhibit transport in MaModBC by preventing the closure of the NBD dimer around ATP. In fact, the only contacts between the two ModC subunits in the trans-inhibited state are formed through the regulatory domains.

The Type I importer structures determined to date have a general theme; open NBDs are coupled with inward-facing conformation of importers and outward-facing conformation of importers will be induced by binding of ATP to the NBDs resulting in closed NBD dimer. Alternation between these two conformations allows movement of substrate across the membrane.

Initiation of the transport cycle is induced by substrate-binding protein

The transport cycle is initiated by the interaction of the SBP with the TMDs. While SBPs were originally divided into three classes according to the arrangement of the core beta-sheets (Fukami-Kobayashi *et al.*, 1999), a growing amount of data lead to a reclassification of SBPs into six clusters based on their three-dimensional structures (Berntsson *et al.*, 2010). Notably, the substrate-binding proteins of structurally characterized Type I importers belong to Clusters B and F (Berntsson *et al.*, 2010). However, predicted Type I transporters such as OppBCDF or PotBCD share operons with SBPs of other clusters including C and D, respectively (Berntsson *et al.*, 2010; Cserzo *et al.*, 2002). It is suggested
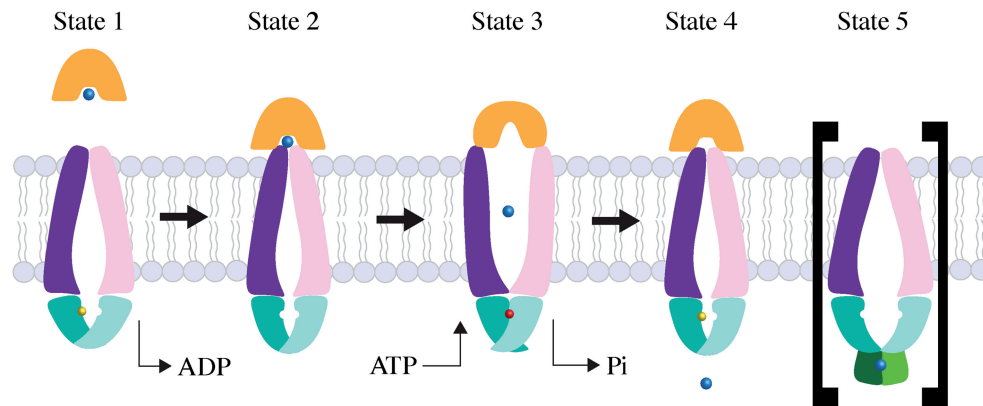
Figure 2. Mechanism of Type I ABC importers. Two TMD subunits are colored purple and pink, and two NBD subunits are colored as cyan and magenta. The periplasmic binding proteins are colored orange and the substrate is represented by a blue sphere. ATP and ADP are shown as a red and yellow sphere, respectively. The substrate-loaded SBP binds to inward-facing conformation of transporter (State 1), and induces a partial closure of two NBD subunits (State 2). Binding of ATP triggers closure of the two NBDs, reorientation of TMD subunits, and opening of the SBP, resulting in release of substrate to the TMD subunits (State 3). The dissociation of ADP molecules resets the transporter back to an inward resting state (State 4). The inhibited state (State 5) is shown in brackets; when cytoplasmic substrate levels are high enough, substrate binds the RDs preventing conformation change and ATP hydrolysis of the transporter.

that most of SBPs consists of two globular domains that are separated by a hinge wherein the ligand is bound and enclosed by an inter-domain rotation (Duan *et al.*, 2001; Hollenstein *et al.*, 2007; Spurlino *et al.*, 1991). Ligand binding brings about a large conformational change in the protein (Sharff *et al.*, 1992; Shilton *et al.*, 1996), and this ligand-induced conformational change appears to be a general feature of the SBPs known to associate with Type I importers. Both the loaded and unloaded forms of MalE can bind to MalFGK (Austermuhle *et al.*, 2004; Davidson *et al.*, 1992). However, only binding of substrate-loaded MalE to transporter can induce the complete closure of two NBDs.

Additional TMD loop contributes to the unique transport process in the maltose transporter

In the maltose transporter, the interaction between MalE and the periplasmic MalF-P2 loop mediates substrate recognition by inducing an activated conformational change of MalE, which precedes substrate transport (Jacso *et al.*, 2012). The P2 loop is a large Ig-like periplasmic domain (P2 loop ~20 kDa) connecting TM helices 3 and 4 and is only observed in the MalF homologues of enteric bacteria (Ehrmann *et al.*, 1998). The P2 loop raps around MalE, forming a receptor that recognizes the SBP and allows MalE and MalF to remain in close contact throughout the catalytic cycle (Daus *et al.*, 2009; Jacso *et al.*, 2009). A structural comparison of P2 loop from the two different states of maltose transporter (Oldham & Chen, 2011; Oldham *et al.*, 2007) indicated that the P2 loop is highly flexible and plays a key role to maintain contacts with MalE regardless of the conformational state of transporter. The flexibility of the P2 loop was further supported by structure of maltose transporter in inward-facing state where flexible P2 loop was not observed in the absence of MalE (Khare *et al.*, 2009).

Post-translational regulation of Type I importers is dependent on the regulatory domain

The regulation mechanism employed by Type I importers is distinct for each transporter, even if they share a similar RD

fold such as MalFGK and MaModBC or transport the same substrate such as AfModBC and MaModBC.

The RD of the maltose transporter contributes to regulatory functions by binding MalT, a transcription factor that is part of the maltose operon, or IIA^{glc}, an enzyme that inhibits transport activity (Boos & Shuman, 1998). Recently, the complex structure of MalFGK with IIA^{glc} revealed that two IIA^{glc} bind the MalK dimer (Chen *et al.*, 2013). This interaction inhibits transport by preventing the closure of the MalK dimer and stabilizing MalFGK in the inward-facing resting state (similar to Figure 2, State 5).

The regulation of MaModBC (Gerber *et al.*, 2008) and MetNI (Kadaba *et al.*, 2008) was accomplished by binding of substrates to the structurally distinct RDs likewise inhibiting closure of the NBD dimer. This indicated that MetNI was regulated through trans-inhibition similar to MaModBC, a conclusion supported by selenomethionine soaking which identified a substrate-binding site in MetN RD (Kadaba *et al.*, 2008). There are two mechanisms known to regulate ModBC. Binding of molybdate directly to the RD as in the MaModBC and repression of *modABC* expression by molybdate-dependent transcription regulator ModE. The substrate-binding C-terminal domain of ModE has similar overall fold as the RD of MaModBC. ModE changes its conformation by binding molybdate (Hall *et al.*, 1999; Schuttelkopf *et al.*, 2003), which allows the N-terminal DNA binding domain of ModE to act as a repressor of the molybdate transporter (Mouncey *et al.*, 1996). This indicates that Type I transporters without RDs like AfModBC (Hollenstein *et al.*, 2007) can be regulated by alternative mechanisms (Grunden *et al.*, 1996).

Type II ABC importers

There are several characteristics that distinguish Type II transporters from Type I. The association of SBP with the Type II transporters is generally weakened by the presence of substrate (Lewinson *et al.*, 2010; Vigonsky *et al.*, 2013). Type II transporters have a greater number of TM helices (Locher, 2009; Oldham *et al.*, 2008), with the core helices

undergoing distinct conformation changes to transport substrate (Korkhov *et al*., 2012b; Rice *et al*., 2013). While Type II transporters share these overall similarities, there is mounting evidence to suggest mechanistic differences within the Type II sub-family (Rice *et al*., 2013, 2014). With substrates ranging from molybdate to vitamin B12, the variety of substrate size may offer some explanation for the observed diversity among Type II transporters.

SBP-mediated uptake in Type II systems

Similar to the Type I importers, substrate specificity in Type II transport systems is defined by SBPs. Interestingly, despite a wide range in substrate size and chemistry, the structurally characterized Type II importers bind to the same class of SBP (Cluster A, Class III; Borths *et al*., 2002; Mattle *et al*., 2010; Tirado-Lee *et al*., 2011). The distinguishing characteristic of Cluster A SBPs is a rigid α-helix in the hinge region connecting two substrate-binding lobes (Berntsson *et al*., 2010). Other SBPs have a flexible hinge region, and substrate-binding forces the lobes to undergo a ''Venus flytrap'' conformation change (Mao *et al*., 1982). Substrate-dependent conformation change in Cluster A SBPs is limited to local loop rearrangement and up to a few degrees of closure (Berntsson *et al*., 2010; Karpowich *et al*., 2003). Even though Cluster A SBPs bind substrates ranging over a 10-fold change in volume, the majority of structural differences between the different SBPs are limited to the loops in the substrate-binding pocket (Tirado-Lee *et al*., 2011).

With only three Type II transporters structurally characterized and limited biochemical data on Type II complexes, it is possible that other types of binding protein may bind with Type II transporters. For example, the Cluster B L-arabinose binding protein ABP (*E. coli* gene name *araF*; Quiocho *et al*., 1974) shares an operon with the putative Type II transporter AraHGp (Scripture *et al*., 1987); AraHp is predicted to have 10 helices in each of the TMDs. Likewise in *Pasteurella haemolytica*, the Cluster D SBP FbpA (Shouldice *et al*., 2004) shares an operon with the putative Type II transporter FbpBCp (Kirby *et al*., 1998), which is predicted to have an 11 TM helix topology. Although the complex formation of ABP to AraHGp or FbpA to FbpBCp has yet to be tested, these cases may broaden the diversity of SBPs in Type II transporter complexes.

Despite limited effects of substrate on SBP conformation in Type II systems, substrates significantly alter the affinity of SBP for Type II transporters (Lewinson *et al*., 2010; Vigonsky *et al*., 2013). In the absence of substrate, Type II transporters form high affinity complexes with their cognate binding proteins. Substrate diminishes this affinity either by increasing the dissociation rate (as for BtuCD; Lewinson *et al*., 2010) or by decreasing the association rate (as for MolBC; Vigonsky *et al*., 2013). The effects of ligand on Type II complex formation are inverted in Type I transporters, which require nucleotide or substrate for complex formation (Vigonsky *et al*., 2013). A close comparison of the affinities of MolA for molybdate and MolBC for MolA has suggested that molybdate weakens complex association through an allosteric binding site on MolBC (Vigonsky *et al*., 2013). However, a substrate-binding site has yet to be observed

for a Type II transporter, though such sites have been reported in Type I transporters (Gerber *et al*., 2008; Oldham *et al*., 2007).

Structural analysis elucidates the diversity and conformational changes in Type II transporters
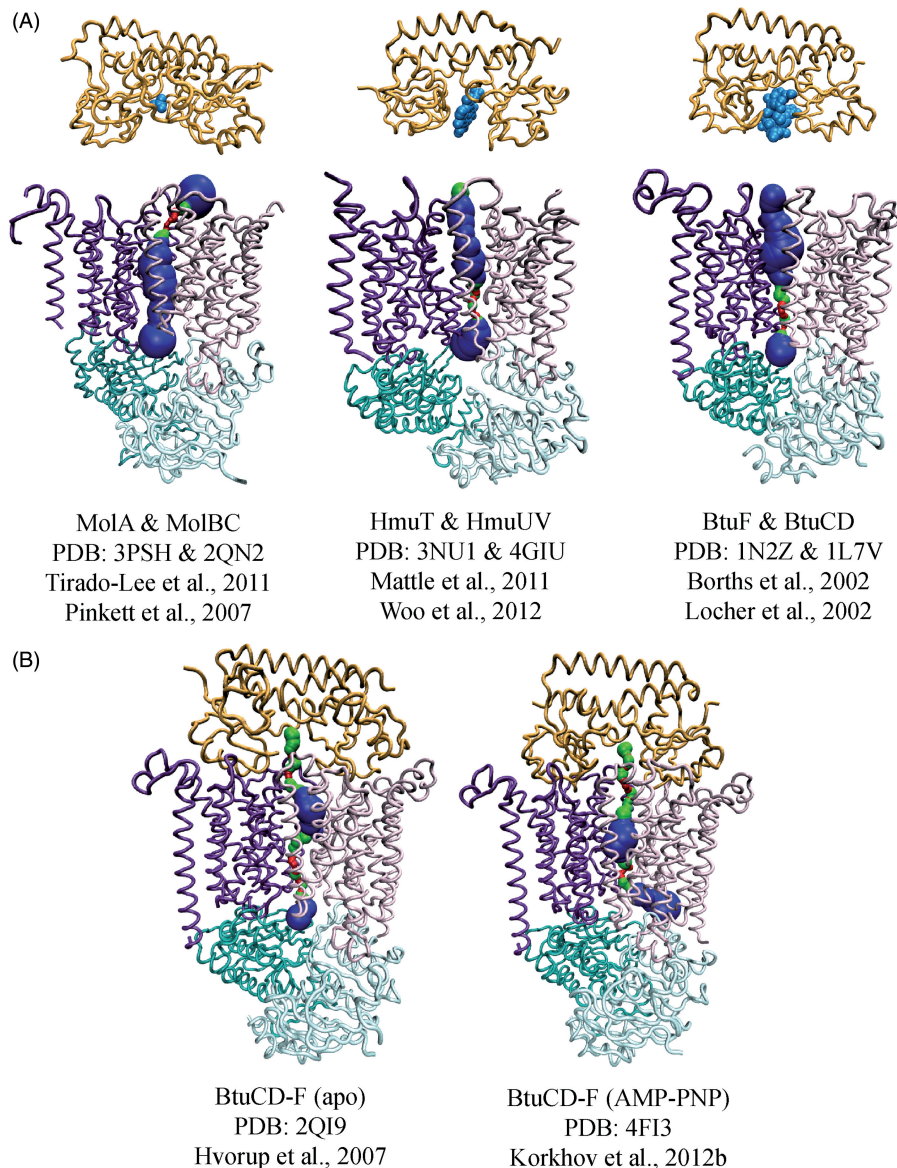
The Type I and II importers were first segregated based on differences in the size and overall architecture of the core transporters (Locher, 2009; Oldham *et al*., 2008). Type II transporters are larger, with the structurally characterized TMD homodimers having 20 TM helices (10 helices per monomer identified as TM 1–10). Close comparison of the TMDs has suggested that the majority of conformation changes are limited to the core helices TM 3, 4, 5, 5a, 10 and the loop between TM 2 and 3. This is distinct from Type I transporters, which undergo domain-wide rigid-body conformation changes as described in the above section.

The core helices control access to the translocation pathway and are central in the transport mechanism. The three structurally characterized Type II core transporters are shown in Figure 3(A) with their cognate SBPs poised on the periplasmic side of the TMDs. The translocation pathway has been highlighted using MolAxis (Yaffe *et al*., 2008), which emphasizes the different conformations of each structure and the accessibility of the translocation pathway. The nucleotide-free conformations of BtuCD and HmuUV are similar showing a central translocation pathway at the TMD interface lined by TM5, 5a, 10 and the TM2-3 loop (Locher *et al*., 2002; Woo *et al*., 2012). In these apo structures, the translocation pathway is in an outward-facing conformation with an occlusion formed by the cytoplasmic side of TM5 (cytoplamic gate I). The key difference between the two transporters is the volume of the translocation pathway. With an internal volume of $\sim$3400 Å^3, BtuCD is able to accommodate vitamin B$_{12}$ with few steric clashes, while HmuUV with an internal cavity of $\sim$1300 Å^3 is able to accommodate one molecule of heme (cavity volumes calculated with 3VEE web server and a large and small probe radius of 10 and 2 Å respectively (Voss & Gerstein, 2010)).

A greater conformational difference is observed with nucleotide-free MolBC, where a closed periplasmic gate (formed by TM 5a) and the open cytoplasmic gate I positions the transporter in the inward-facing conformation (Pinkett *et al*., 2007). It is notable that access through the periplasmic gate appears to correlate with the size of substrate, suggesting that the translocation pathway may add to substrate selectivity with the same filter mechanism as a channel.

BtuCD has also been solved in complex with its SBP BtuF in the presence (Korkhov *et al*., 2012b) and absence (Hvorup *et al*., 2007) of nucleotide (Figure 3B), which provides us with information on the conformational flexibility of a Type II importer. The nucleotide-free BtuCD-F complex displayed an asymmetric conformation of TM5 and 5a, with one TMD subunit resembling the outward-facing, un-complexed BtuCD and the other subunit resembling the inward-facing MolBC (Hvorup *et al*., 2007). This conformation of the translocation pathway was occluded, leaving inadequate space for substrate. In the presence of nucleotide, a large internal cavity was formed between a closed periplasmic gate and a closed

Figure 3. Conformational diversity among Type II importer crystal structures with MolAxis prediction of the translocation pathways. The TMD subunits of each transporter are shown as dark purple and pink tubes, the NBD subunits are teal and cyan, the SBPs are orange, and substrates are light blue. The MolAxis program was used to find and analyze the translocation pathway by fitting the largest possible spheres in along the central cavity (Yaffe *et al.*, 2008). Spheres with a diameter <1.4 Å are colored red, 1.4–4 Å green and >4 Å blue. (A) Type II core importers MolB$_2$C$_2$ from *H. influenza*, HmuU$_2$V$_2$ from *Y. pestis* and BtuC$_2$D$_2$ from *E. coli* each solved in the absence of nucleotide. Cognate SBPs (MolA, HmuT and BtuF, respectively) are shown poised above the periplasmic gates. (B) Type II importers solved in complex with SBP. The BtuCD-F structure on the right was solved with AMP-PNP bound (nucleotide not shown).

(A)

MolA & MolBC
PDB: 3PSH & 2QN2
Tirado-Lee et al., 2011
Pinkett et al., 2007

HmuT & HmuUV
PDB: 3NU1 & 4GIU
Mattle et al., 2011
Woo et al., 2012

BtuF & BtuCD
PDB: 1N2Z & 1L7V
Borths et al., 2002
Locher et al., 2002

(B)

BtuCD-F (apo)
PDB: 2QI9
Hvorup et al., 2007

BtuCD-F (AMP-PNP)
PDB: 4FI3
Korkhov et al., 2012b

second cytoplasmic gate (gate II) formed by the TM2–3 loop. Although, substrate was not present in the structure, the cavity is large enough to hold vitamin B12 with few steric clashes (Korkhov *et al.*, 2012b).

Mechanistic details of the Type II importer, BtuCD

Structural snap-shots, biochemical analysis and electron paramagnetic resonance (EPR) spectroscopy of BtuCD-F have culminated in a well-defined Type II transport mechanism. Here, the mechanism can be described as four states. Analysis of complex association rates in an excess of ATP-Mg^{2+} has suggested that the majority of a BtuCD transporter population can be found in the ADP-bound state (Figure 4A, State 1; commonly known as the post-hydrolysis state; Lewinson *et al.*, 2010), which has a nearly identical conformation to the nucleotide-free transporter (Joseph *et al.*, 2011, 2014). With the periplasmic gate (TM 5 and 5a) open and cytoplasmic gate I (TM5) closed, the nucleotide-free state is outward-facing (Locher *et al.*, 2002). Substrate-loaded BtuF binds to BtuCD, delivering substrate to the

outward-facing translocation pathway (Figure 4A, State 2; Borths *et al.*, 2005; Locher *et al.*, 2002).

The BtuCD-F complex has very little affinity for vitamin B12 (Borths *et al.*, 2005; Lewinson *et al.*, 2010), and substrate transfer may be encouraged by a slight opening of the BtuF lobes and loops from the periplasmic gate reaching toward the substrate-binding pocket (Hvorup *et al.*, 2007). The ADP bound transporter, with a closed cytoplasmic gate I, prevents passage of substrate to the cytoplasm (Goetz *et al.*, 2009; Joseph *et al.*, 2011). When BtuCD-F binds nucleotide (Figure 4A, State 3), the NBDs close, pulling the coupling helices closer together. This triggers the closing of the periplasmic gate and concomitantly, the opening of cytoplasmic gate I and closing of cytoplasmic gate II, which traps substrate in a large translocation chamber (Korkhov *et al.*, 2012b; Joseph *et al.*, 2011, 2014).

Studies with ATP analogs representing the pre-hydrolysis ATP bound state (ATP + EDTA, AMP–PNP) and the intermediate state before phosphate departure (ADP + Mg^{2+} + vanadate) show similar conformations. This suggests that
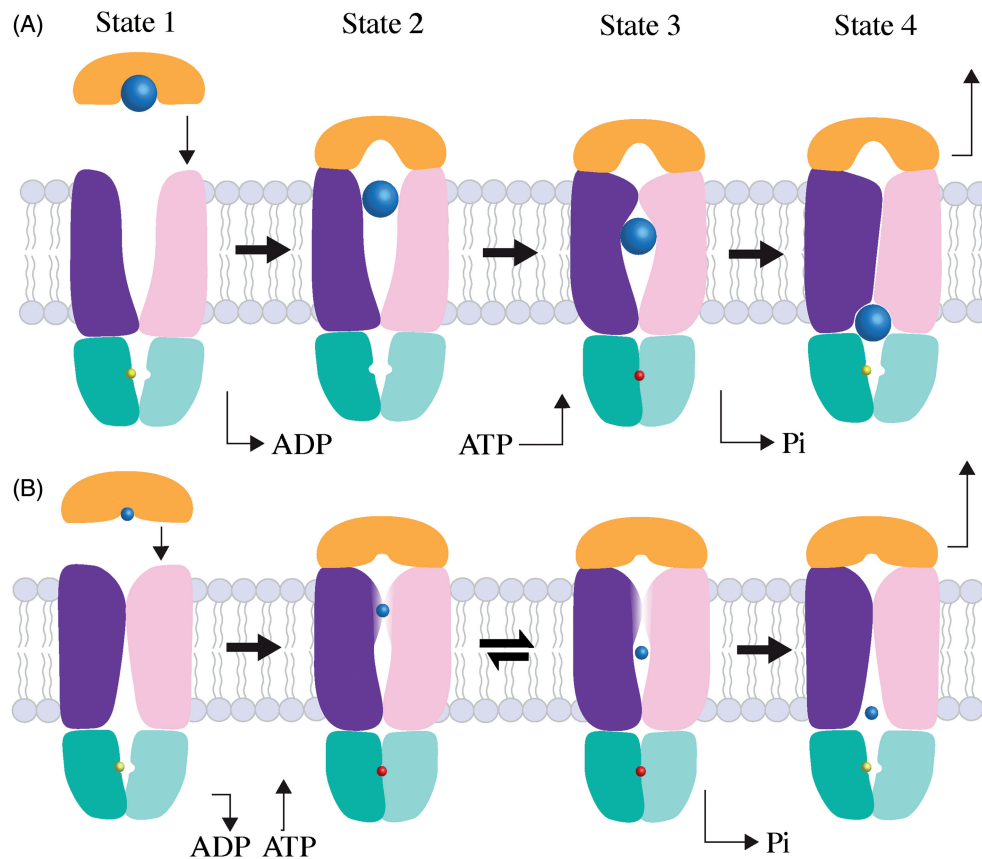
Figure 4. Mechanisms of large and small substrate Type II transporters. The TMD subunits are colored dark purple and pink, the NBD subunits are teal and cyan, the SBP is orange and substrates are light blue. The colors of TMD, NBD, SBP and substrate are the same as in Figure 3. (A) Transport of a large substrate is initiated when substrate-loaded binding protein docks to the periplasmic surface of the transporter. In State 1, the transporter is shown with ADP (yellow) bound, but the nucleotide-free state is conformationally identical. In State 2, we show SBP docking before ATP due to the higher affinity of transporter for loaded SBP in the absence of nucleotide (Lewinson *et al.*, 2010) and the slightly more open conformation of the BtuCD-F periplasmic gate in the absence of nucleotide (Joseph *et al.*, 2011). State 3 shows that the binding of ATP (red) traps substrate (blue) in a translocation chamber. Post-hydrolysis, the NBDs open allowing the departure of phosphate and the concurrent collapse of the translocation chamber. Substrate is depicted as it is being squeezed into cytoplasm by the transporter progressing toward an occluded complex (State 4). The transport cycle restarts when the occluded complex disassembles. (B) Transport of a small substrate is initiated when loaded SBP binds to the transporter (State 1). Though small substrate has yet to be trapped in a Type II complex, we suspect that substrate cannot be transferred until ATP binds and the periplasmic gate opens (State 2). After transfer, small substrate may be held in an internal cavity comparable to large substrates (State 3). The possibility of reversion from State 3 to State 2 cannot be ruled out, and may be distinct from the sealed cavity of large substrate Type II transporters. After nucleotide is hydrolyzed, the small substrate transporter returns to an apo-like inward-facing state, from which substrate can enter the cytoplasm (State 4).

post-hydrolysis conformation changes coincide with phosphate departure (Joseph *et al.*, 2014). When nucleotide is hydrolyzed, the NBDs separate, phosphate departs, cytoplasmic gate II opens and the translocation chamber collapses with the TM5 helices squeezing vitamin B12 into the cytoplasm (Joseph *et al.*, 2014). After the departure of substrate, BtuCD-F is in a stable, occluded conformation (Figure 4A, State 4; Hvorup *et al.*, 2007, Korkhov *et al.*, 2012a). The mechanism for disassociation of the Type II transporter complex is not well understood, but Lewinson *et al.* (2010) have suggested that basal ATP hydrolysis and possibly free substrate may play important roles (Vigonsky *et al.*, 2013).

Mechanistic diversity among Type II transport systems

Studies of the molybdate importer MolBC have uncovered novel characteristics of the Type II transporter mechanism. EPR and cross-linking studies of MolBC-A confirmed the nucleotide-free, inward-facing crystal structure of MolBC (Pinkett *et al.*, 2007; Rice *et al.*, 2013, 2014; Figure 4B, State 1). In MolBC, the nucleotide-dependent movement of the periplasmic gate is more limited than BtuCD, with TM 5a shifting apart enough to disrupt disulfide cross-linking. This increases the mobility of spin-labels along the helix and presumably allows the passage of a relatively small substrate like molybdate (Rice *et al.*, 2013). In the presence of nucleotide, we suspect that substrate passes from the SBP into the translocation pathway (Figure 4B, State 2; Rice *et al.*, 2013). When nucleotide is bound to the transporter, the cytoplasmic ends of the TM 5 helices shift away from each other and do not assume a closed conformation, and thus this region of MolBC cannot be considered a gate (Rice *et al.*, 2013). The outward movement of TM 5 is likely coupled to the inward movement of cytoplasmic gate II, which closes in the presence of nucleotide. The resulting conformation (Figure 4B, State 3) is predicted to be similar to that of BtuCD-F (Figure 4A, State 3), with substrate trapped in the

Figure 5. Domain architecture and assembly of Group I and II ECF transporters. ECF transporters contain an energy module (A and A′, colored in teal and cyan respectively), transmembrane component (T, colored in purple) and a substrate-binding module (S, colored in light purple, light blue and light pink). In Group I ECF transporters, S associates with dedicated T, A, A′ units, while Group II ECF transporters utilize the same T, A and A′ components for several different S components.
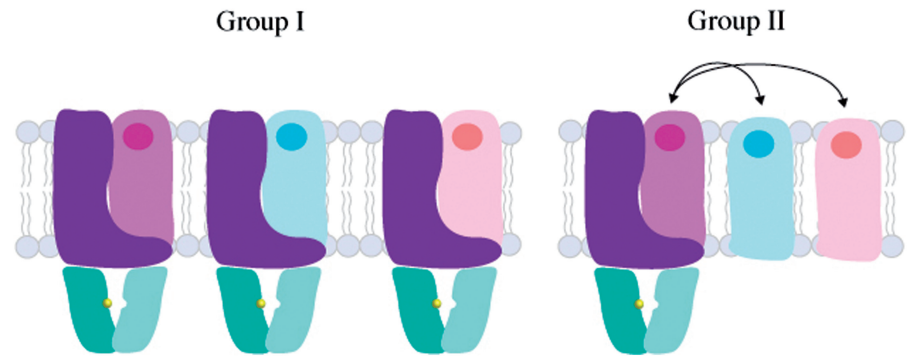
translocation chamber. (Rice *et al*., 2013, 2014). For MolBC-A, there is currently no evidence to support an occluded conformation. Instead hydrolysis of ATP returns the transporter to an apo-like inward-facing state (Figure 4B, State 4), where substrate is allowed to enter the cytoplasm (Rice *et al*., 2013). It is possible that the mechanistic differences between MolBC and BtuCD can be traced back to the size of substrate, where a large opening of the periplasmic gate was not required for molybdate passage. And, with the periplasmic gate closed in the nucleotide-free conformation, cytoplasmic gate I was not required (Rice *et al*., 2013). It remains to be seen if the MolBC mechanism will be observed for other small-substrate Type II importers or if additional variations on the Type II transporter mechanism will be found.

Given the differences in substrates, it should come as no surprise that the structures and mechanisms of Type II transporters have diversified. As we continue to explore the Type II subfamily, we can expect to find additional sources of diversity. For Type II transporters, the biggest questions remain in the area of regulation. Whereas Type I transporters have well-described mechanisms of transcriptional and post-translational regulation, regulation of Type II transport is largely unexplored. Like Type I transporters, Type II systems can be regulated by substrate at the transcription level [MolBCA (Rice *et al*., 2014), HmuUVT (Chao *et al*., 2005) and FhuBCD (Andrews *et al*., 2003)]. A mechanism of post-translational regulation for a Type II system has yet to be verified, but a thorough database search (http://exon.niaid.nih.gov/abctransporter/) can reveal extensions to the core transporter that may prove to be Type I-like RDs or other accessory domains.

The emergence of ECF transport system: Type III transporters?

Energy-coupling factor (ECF) transporters were first discovered in the 1970s (Henderson *et al*., 1979), but only recently classified as a new type of ABC transporter (Rodionov *et al*., 2009). These proteins play a critical role in micronutrient uptake in bacteria and archaea. Similar to Type I and II transporters, ECF transporters consist of two nucleotide-binding domains (EcfA and EcfA′), a transmembrane coupling domain (EcfT) and a substrate-binding component (EcfS). EcfA components can assemble as homodimers or heterodimers and the EcfA–EcfA′–EcfT complex forms the energizing module. While Type I and II

ABC importers have a substrate-binding protein to deliver compounds to the translocation pathway, ECF transporters contain a unique feature: the EcfS component. The EcfS, or S component, is an integral membrane protein that binds substrate with nanomolar affinity (Duurkens *et al*., 2007; Hebbeln *et al*., 2007). S components also differ in their genetic organization and the way they interact with the energy module, separating ECF transporters into two subgroups (Figure 5). For Group I ECF transporters, each S component forms a complex with a unique energizing module and the S-components are located within the same operon. Alternatively, Group II ECF transporters consist of modular S components that bind to a diverse array of substrates, yet multiple S components share the same energizing module to make up the full transporter (S, T, A and A′). For Group II ECF transporters, the S components are scattered across the genome. All three energizing components (T, A, A′) are necessary for the S component to form a complex, and all four components (T, S, A and A′) of the ECF transporter are necessary for transport of substrate across the membrane (Karpowich & Wang, 2013). Despite the diversity of substrates and assembly that differentiate these transporters from Type I and II importers, ECF transporters also use the energy of ATP to translocate substrates across the lipid bilayer.

The S component is responsible for substrate binding

From thiamine to riboflavin, S components bind a diverse range of substrates. To date, structures of the S components ThiT (thiamine; Erkens *et al*., 2011) and BioY (Biotin) from *Lactococcus lactis* (Berntsson *et al*., 2012), RibU (riboflavin) from *Staphyloccus aureus* (Zhang *et al*., 2010) and NikM (nickel) from *Thermoanaerobacter tengogensis* (Yu *et al*., 2014) have been solved (Figure 6A). In addition, the complete complex structures of the Folate (Xu *et al*., 2013) and hydroxymethyl pyrimidine (HMP) (Wang *et al*., 2013) transporters from *Lactococcus brevis* were solved in the absence of substrate (Figure 6B).

While not sequentially similar, the structures of RibU, BioY and ThiT reveal that helices 4, 5 and 6 directly interact with the riboflavin, biotin and thiamine, respectively. The long L1 loop between helices 1 and 2 covers the substrate-binding pocket in the substrate bound structures, but in the full length structure, this loop does not cap the binding pocket, potentially indicating a substrate sensing mechanism. Understandably, there is variation in sequence amongst the

(A)   (B)

RibU (Riboflavin)
PDB:3P5N
Zhang *et al.*, 2010

ThiT (Thiamine)
PDB:3RLB
Erkens *et al.*,2011

BioY (Biotin)
PDB:4DVE
Berntsson *et al.*, 2012

NikM2 (Nickle and colbalt)
PDB:4M5B
Yu *et al.*, 2014

Folate ECF transporter
PDB:4HUQ
Xu *et al.*, 2013

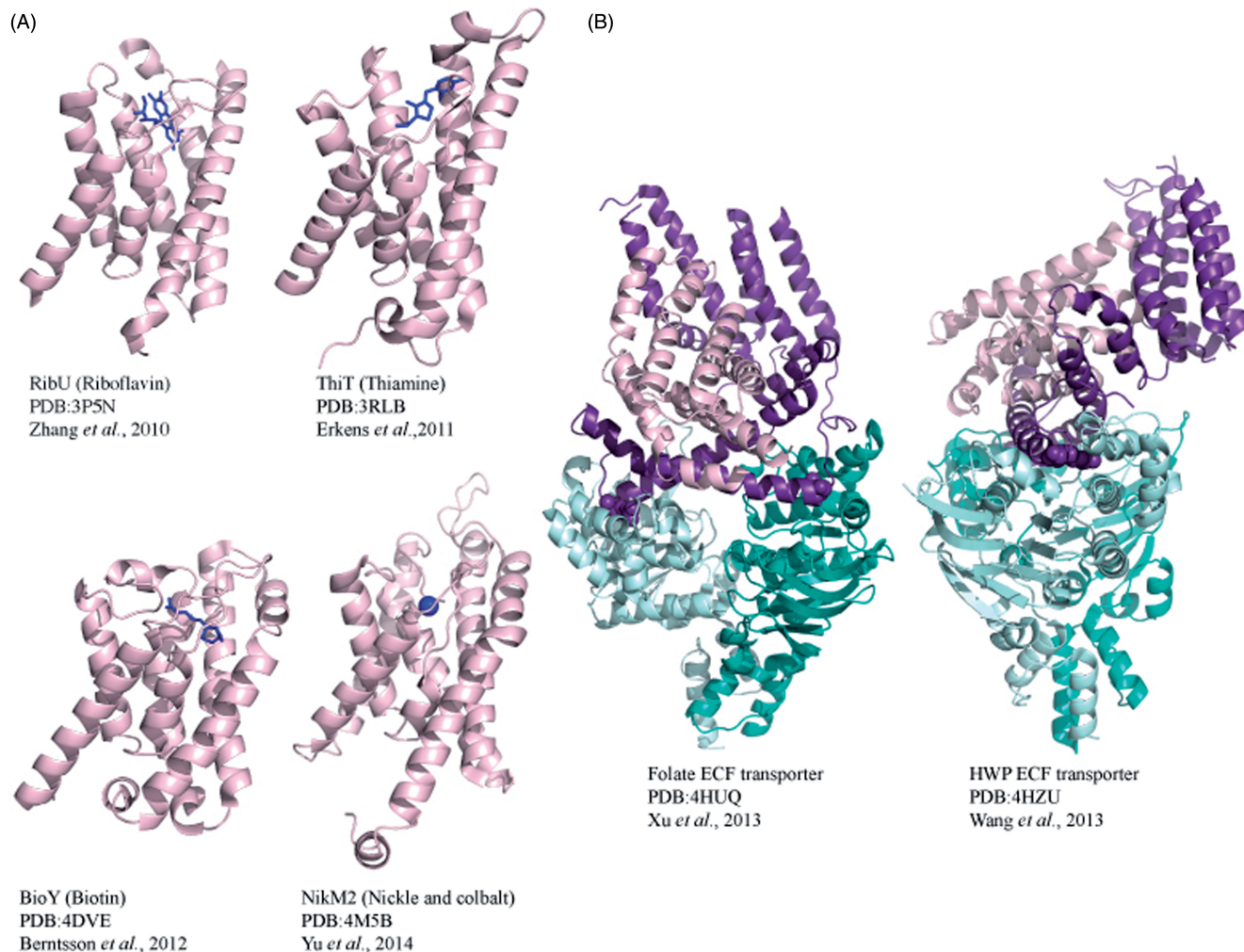HWP ECF transporter
PDB:4HZU
Wang *et al.*, 2013

Figure 6. Crystal structures of Type III ECF importers. (A) Cartoon diagrams of S component crystal structures RibU from *S. aureus*, ThiT and BioY from *L. lactis* and NikM2 from *T. tengcongensis*. Each S component is bound to substrate shown in blue stick representation. (B) Crystal structures of full-length, substrate-free folate and HMP transporters from *L. brevis*. For both full-length structures, the energizing component (shown in purple) predominantly interacts with the substrate bound component in pink. The EcfA and EcfA′ are shown in cyan and teal.

EcfS binding pocket that accounts for specificity, but the S components have substrate affinity in the nM to pM range. NikM, the S component of the ttNikM2ECF transporter from *Thermoanaerobacter tengcogensis* (Yu et al., 2014) fits into a subclass of S component metal transporters that are part of the Group I transport system. Similar to the nutrient EcfS components, NikM contains the core 6 helices and has two additional modifications that make this group unique. The first nine residues that make up a loop proceeding helix 1 are conserved across ECF metal S components. This N-terminal loop would block the vitamin binding sites found in other S components. In combination with an additional N-terminal helix, both form a binding site specific for metals that would prevent vitamin binding (Yu *et al.*, 2014).

Full-length structures shed light on assembly and mechanism of ECF transporters

The most recent structures of the Folate and HMP ECF transporter from *L. brevis* (Wang *et al.*, 2013; Xu *et al.*, 2013) have provided us with a glimpse into the assembly and mechanism of this class of transporters. These full structures

provide the first view of the EcfT domain and how the ECF components assemble to make an intact ECF transporter (Figure 6).

The EcfT domain consists of eight helices, five of which make up the EcfT core domain perpendicular to the membrane and additional helices dipping into the cytoplasmic space, which form an ''L'' shape configuration. The full-length structures in the absence of substrate reveal how the S component interacts with the energizing component EcfT. The S component fits between the five core helices and cytoplasmic helices, with five of the six EcfS helices making contacts with EcfT. In complex, helices 1, 2 and 6 of EcfS are responsible for most of the hydrophobic contacts with EcfT. The structures are supported by studies on the conserved motif (AXXXA, X mostly hydrophobic) in helix 1 shown to be essential for interactions with the ECF energizing module (Berntsson *et al.*, 2012; Erkens *et al.*, 2011).

Structurally different from the configuration of Type I and II transporters, the cytoplasmic EcfT helices (but not the EcfS helices) make direct contacts with EcfA and EcfA′. EcfT helices 6 and 7 both contain a conserved sequence called the coupling module, XRX, which directly interacts with a groove

in each of the nucleotide binding domains. Double mutations of the conserved arginine residues result in a dissociation of the ECF complex and loss of ATPase activity (Neubauer *et al.*, 2009). Coupling helices are also present in ABC importers and exporters, as a means of communication between the TMD and NBD (Locher, 2009).

The dimerized structure of the EcfA–EcfA′ in the full length structures is similar to the structures of homodimer CbiO of CbiMNQO the cobalt EcfA–EcfA′ energy module from *Thermoanaerobacter tengcongensis* (Chai *et al.*, 2013) and EcfA–EcfA′ heterodimer structure from *T. maritima* (Karpowich & Wang, 2013). Similar to Type I and II transporters, the NBD is made up of a helical subdomain and RecA-like domain with the conserved motifs for ATP binding and hydrolysis. The structures of the ECF transporter show two C-terminal helices, which form a four-helix bundle upon dimerization not common to all ABC importers.

Although both full-length ECF structures are substrate and nucleotide free, they start to provide insights into a possible mechanism. The EcfA–EcfA′ conformation is most similar to a nucleotide free open conformation, leading one to believe that the structure is currently in an inward-facing conformation. For both Group I and II ECF transporters, it is assumed that ATP induced conformational changes are coupled to the substrates translocation and subsequent release into the cytoplasm. The structures reveal that the S component of the ECF transporter lies parallel to the membrane surface, presumably in a conformation to allow for substrate release into the cytoplasm. In both full-length structures, the angle of the S component makes the opening of the substrate-binding pocket accessible to the lipid–cytoplasm interface. Based on this conformation, the proposed mechanism would begin with the nucleotide free resting state, where the EcfS component is perpendicular to the EcfT component in the membrane and the EcfA–EcfA′ components are far apart. ATP binding would bring EcfA–EcfA′ closer together into a closed conformation and rotate the EcfS module into a parallel position so it could bind extra-cellular substrate. ATP hydrolysis would rotate the ECF transporter back to the resting state for subsequent substrate release in the cytoplasm. As we go forward with these Type III ABC importers, the major questions will include connecting substrate translocation to nucleotide states and the conformational changes necessary to trigger the release of substrate. We await the substrate-loaded and nucleotide-bound full-length structures of ECF transporters to shed light on the proposed mechanisms.

Conclusion

The culmination of genetic, structural and biochemical studies of ABC importers to date have revealed three different systems that utilize ATP to transport an array of substrates across cellular membranes. While the overall architectural differences between Type I, II and III transporters reflect diversity in both the mechanism of transport and regulation, these systems only scratch the surface on the complexity of this family. ABC importers have evolved to use multiple mechanisms not only to transport nutrients across the membrane but also to survive in an ever-changing and hostile environment (Kjelleberg *et al.*, 1993; Lewis *et al.*, 2012; Lin *et al.*, 2009).

Genome analysis uncovered multiple importers that transport the same substrate within an organism. Studies of the *mol* and *mod* systems in *H. influenzae* revealed the co-existence of a low and high affinity transport system for molybdate in the same organism (Rice *et al.*, 2014). This duplication is also seen in *E. coli*, albeit with less specificity for one substrate. Here, *cys, mod* and an additional nonspecific transport system are responsible for molybdate and sulfate transport (Aguilar-Barajas *et al.*, 2011; Sirko *et al.*, 1995). In several bacterial organisms, the presence of two sulfate transport systems are found in separate operons, suggesting an importance of redundancy in uptake systems for particular bacteria (Aguilar-Barajas *et al.*, 2011).

We are also finding unexpected diversity in the substrates bound by particular SBPs and transport systems. The sap operon (*sapABCDFZ*) from non-typable *Haemophilus influenzae* (NTHI) encodes for importers that select for antimicrobial peptides (AMPs) for transport into the cytoplasm and subsequent degradation, presumably before peptide accumulation can cause irreparable damage to the cytoplasmic membrane. The SapA binding protein has the ability to select for both heme and AMPs, present in different microenvironments in the host (Mason *et al.*, 2011; Shelton *et al.*, 2011). This mechanism also seems to be conserved in other bacteria, which need to survive under different environmental stresses (Tanabe *et al.*, 2007; Vanderlinde *et al.*, 2010). These systems not only suggest diversity in substrate recognition but also possible variation in the complexes formed between SBP and transmembrane domains.

While it was once thought that ABC importers utilized a single SBP per transporter located on the same operon, we now see examples of multiple SBPs per transporter complex. For example, GlnPQ importer from *E. faecalis* and *S. pneumonia* has multiple SBPs with high sequence similarity, yet each differ in their affinity to glutamine. In addition these SBPs can also bind asparagine with low affinity (Fulyani *et al.*, 2013), reflecting the diversity introduced through the SBPs that interact with the same transport system.

As we begin to structurally and functionally elucidate additional transport systems with an overall architecture that differs from what is currently known, there is the potential to discover an even greater diversity among these complex systems. Going forward, our comprehension of transport systems will depend not only on understanding the details of what distinguishes a Type I importer from a Type II or the role of size of substrate in the mechanism of transport, but also an overall analysis of the transport systems in a given organism. The multiplicity in transport components, whether it is duplicate transporters or multiple SBPs that interact with the same transporter make these systems multifunctional. Since nutrient uptake is interconnected to bacterial survival and pathogenesis, a detailed understanding of the interplay between transport systems will be essential.

Declaration of interest

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