Chapter 1

ABC Transporters

Transport Proteins

Approximately 10% of all the proteins in a cell are transport proteins that move substrates from one side of a biological membrane to the other (1, 2). Saier and colleagues have compiled a list of all known transport proteins and classified them based on their mechanism of transport and energy source (3). The three largest categories are channels/pores, electrochemical potential-driven transporters and primary active transporters (Figure 1).

Channels and pores. Channels and pores provide an aqueous pathway through which molecules can flow down their concentration gradient. This category comprises alpha-helical membrane spanners such as voltage and ligand-gated ion channels, porins having a beta-barrel membrane spanning region, and also pore-forming toxins.

Electrochemical potential-driven transporters. Electrochemical potential-driven transporters use the energy stored in chemiosmotic gradients to move substrates both along and against their concentration gradients. Included in this category are the so-called porters including those of the major facilitator superfamily. Major facilitators are found in all organisms and are specific for a wide range of substrates including sugars, ions, drugs and neurotransmitters. They can catalyze uniport (facilitated diffusion of a single solute down its concentration gradient), symport (in which two solutes are moved in the same direction, one along and one against its concentration gradient) and antiport (in which two solutes move in opposite directions, one along and one against its

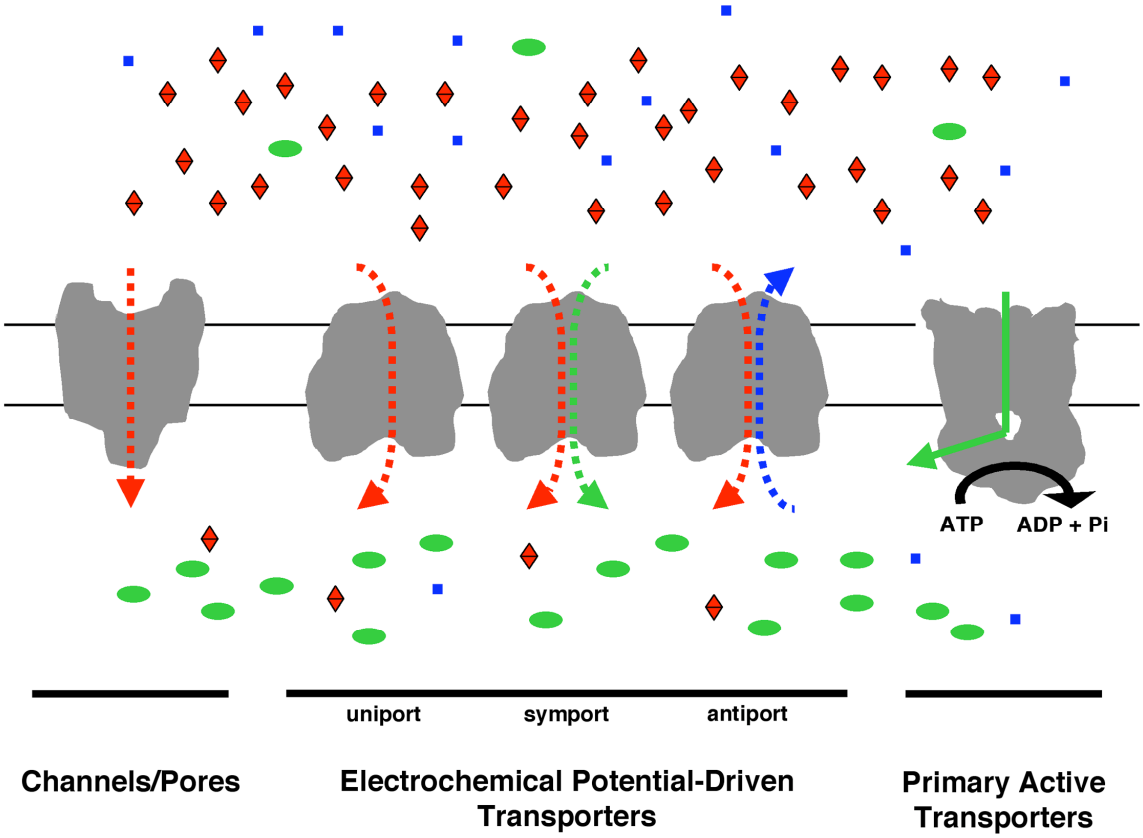

Figure 1. Transport proteins. Three main classes of transport proteins are illustrated. Arrow color and orientation represent substrate type and direction of transport.

concentration gradient). Another protein in this category is the TonB complex of gram-negative bacteria that can use the power of the proton motive force to energize otherwise passive outer membrane porins to accumulate substrates against a concentration gradient.

Primary active transporters. Transporters in the third category are called primary active transporters because they can move substrates against a concentration gradient and are powered by a primary energy source (electrical, chemical or solar). One subclass, the light absorption-driven transporters, harnesses the energy of the sun to move ions across a membrane (e.g., bacteriorhodopsin, halorhodopsin). A second subclass of primary active

transporters are diphosphate bond hydrolysis dependent transporters which use the chemical energy stored in the P-P bond of inorganic phosphate, ATP or other nucleoside triphosphates to power substrate translocation. Included in this subclass are the Sec family protein translocases, the P-type ATPases (including the Na^+/K^+ ATPase) and ATP-binding cassette (or ABC) transporters.

ABC Transporters

ABC transporters are a very large family of membrane transport proteins that move substrates across cellular and organelle membranes (4-8). Using the power of ATP binding and hydrolysis, these proteins can pump substrates both into and out of the cytoplasm, against a concentration gradient (Figure 2). Nearly ubiquitous in nature, ABC transporters are found in bacteria, Archaea, plants and animals. They have roles in nutrient and micronutrient uptake, osmotic regulation, antigen processing, and toxin/drug export. ABC transporters are also very clinically relevant. Currently, they are implicated in thirteen different genetic diseases in humans (9). One well-known example, cystic fibrosis, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), which functions as a chloride channel. The eukaryotic ABC transporter P-glycoprotein/MDR1 is capable of exporting hydrophobic drugs from cells, thus contributing to the resistance of tumor cells to chemotherapeutic agents (4).

Molecular and genetic architecture. All ABC transporters possess a similar molecular architecture: two membrane spanning domains (MSDs) that form a substrate

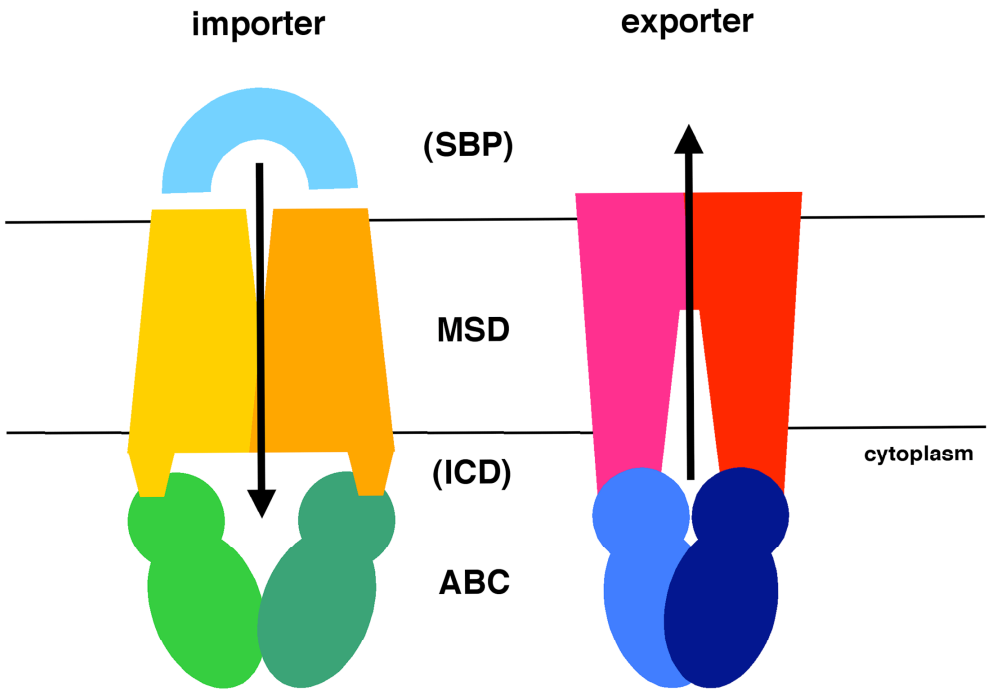

Figure 2. Molecular architechture of ABC importers and exporters. The two membrane spanning domains (MSDs) of the importer are colored light and dark orange, the substrate binding protein (SBP) is light blue and the two ATP-binding cassette (ABC) domains are light and dark green. The MSDs of the exporter are pink and red while the ABC domains are light and dark blue. The extention of the exporter MSDs into the cytoplasm in this cartoon represents the intracellular domain (ICD).

translocation pathway and two peripherally associated ATP-binding cassette (ABC) subunits that bind and hydrolyze nucleotide to power substrate translocation (Figure 2) (4, 5, 8). In contrast, the genetic architecture of transporters can vary from four separate genes (one encoding each subunit) to one gene encoding a half-transporter (one MSD plus one ABC) to one gene encoding an entire transporter (10). The prior arrangements are common in bacteria while the latter is present only in eukaryotes. In general, all the genes for one ABC transport system reside within one operon and can be co-regulated, though there are exceptions. Phylogenetic studies have shown that ABC transporter operons probably evolved together with little shuffling of components between transport

systems. It is thought that importers and exporters diverged from each other before the separation of eukaryotes from prokaryotes. While both eukaryotes and prokaryotes have exporters, only prokaryotes have retained importers.

The substrates transported by ABC transporters range from single ions to polypeptides and hydrophobic drugs to sugars (8), a diversity which is reflected in the poor sequence similarity between the membrane spanning domains of various transporters (11). Indeed, various transporters are predicted to have anywhere from 10 to 20 membrane spanning helices (4, 12). Unlike MSDs, ABC domains are characterized by several highly conserved sequence motifs, including the P loop/Walker A, Walker B, Q loop, switch region and ABC signature sequence motifs, all of which are involved in the binding and hydrolysis of nucleotide (7). This conservation of architecture suggests that there is also a conservation of function and mechanism in the family of ABC transporters.

Eukaryotic and bacterial exporters. Most eukaryotic ABC transporters are exporters that move substrates from the cytoplasm outside the cell or into an intracellular compartment (3, 6). As mentioned above, several families of multidrug efflux pumps have been identified in humans that can transport a wide range of hydrophobic and amphiphilic compounds from the cytoplasm or plasma membrane out of the cell. The TAP transporter moves peptides from the cytoplasm into the endoplasmic reticulum for binding to MHC molecules and is thus vital for immune system function. Other examples include transporters for iron, retinal, bile salts and fatty acids.

Bacteria also have ABC type exporters. LmrA, a transporter resembling P-glycoprotein/MDR1, is found in *Lactococcus lactis*, and has been shown to be a multidrug exporter (13). Exporters also have other functions in bacteria, including secretion of cell surface components, antibiotics, siderophores and the flipping of lipids from one leaflet of the bilayer to the other (7).

Bacterial binding protein-dependent importers. Most bacterial ABC transporters are nutrient importers with diverse substrates including sugars, amino acids, iron siderophores, peptides, ions and osmolytes (3). In addition to having two MSDs and two ABC domains, bacterial importers generally have a cognate substrate binding protein (SBP) that delivers the substrate to the translocation channel (Figures 2 and 4) (5, 14, 15). In gram-negative bacteria like *E. coli*, SBPs are separate, soluble proteins located in the periplasmic space. In gram-positive bacteria, they can be attached to the cell membrane via a lipid anchor (16), while Archaea can use an N-terminal transmembrane helix (17, 18). Two other families of SBPs are actually fused directly to the membrane spanning domain of their cognate transporter (18). A SBP is an integral part of its cognate transporter system and uptake by wild type transporters is dependent upon them (19, 20). They bind their substrates with high affinity and selectivity, helping ensure, and in some cases determine, the specificity of its cognate transporter (14, 21).

SBP-dependent uptake systems that have been functionally characterized include the maltose transporter of *E. coli*, the histidine permease of *S. typhimurium*, and the glycine-betaine transporter of *L. lactis*.

Structural Characterization of ABC Transport Systems

Substrate binding proteins. The first components of bacterial ABC transport systems to be structurally characterized were periplasmic SBPs. Despite very little sequence conservation, there is a remarkable conservation of structure in these proteins (22). All are bi-lobed proteins that bind their substrate at the interface of the two sub-domains. Each sub-domain generally consists of a beta sheet surrounded by alpha helices. Some SBPs completely enclose their substrates, while other bind their ligands in more solvent-accessible sites (22-25). SBPs have been divided into three groups based upon their inter-domain connectivity (22, 24). In Group I, three flexible beta-strands connect the sub-domains; Group II binding proteins have two flexible beta-strand linkers; and Group III SBPs have a single, rigid alpha helical connection. In Group I and II SBPs, the presence or absence of substrate can have a profound effect on the relative orientation of the two sub-domains (22). The structures of two Group III SBPs in the presence and absence of substrate, however, reveal that no large hinge or twist motions occur in this class of SBPs upon ligand binding/release (25, 26).

ABC domains. The structures of isolated ABC domains from multiple transport systems have also been solved by x-ray crystallography, including examples of both importers and exporters, as well as bacterial and eukaryotic proteins (7). As noted above, there is significant sequence conservation between ABC domains from various transport systems and these crystallographic studies confirm that their structure is also conserved. Every ABC domain has two sub-domains: an alpha-beta sub-domain similar to that found in the

RecA protein, containing the Walker A, Walker B and switch motifs, as well as a smaller helical sub-domain that is unique to ABC transporters and contains the ABC signature sequence. The Q loop motif forms the connection between the alpha-beta and helical sub-domains. Though there was some initial confusion, it is now clear that ABC domains dimerize such that two ATP binding sites are formed at the dimer interface with the Walker A motif of one subunit and the ABC signature motif of the other contacting one ATP molecule (7, 27).

Complete transporters: importers. The structure of one complete bacterial importer, BtuCD, has been solved by x-ray crystallography (Figure 3) (12). In conjunction with its cognate binding protein, BtuF, BtuCD transports vitamin B_{12} in *E. coli*. The structure of BtuCD revealed a translocation pathway formed at the interface of two BtuC membrane spanning domains with a single closed gate near the cytoplasmic surface. Each BtuC subunit consists of 10 membrane spanning alpha helices arranged in an intricate pattern. Two short alpha helices (L1 and L2), designated the L-loop, are formed by cytoplasmic loops of BtuC. The two BtuD ABC subunits are positioned below the BtuC subunits and arranged "head to tail" to form two ATP binding sites at the dimer interface, as expected from the structures of isolated ABC domains. Residues surrounding the Q loop of BtuD make contact with the L-loop of BtuC. This region is proposed to be the transmission interface for signaling between BtuC and BtuD (12, 27). There is a large, water-filled hole in the middle of the four subunits through which the transported B_{12} molecule probably escapes after passing through the translocation channel. The structure of BtuF, BtuCD's cognate SBP, is described in Chapter 2 and Appendices 1 and 2.

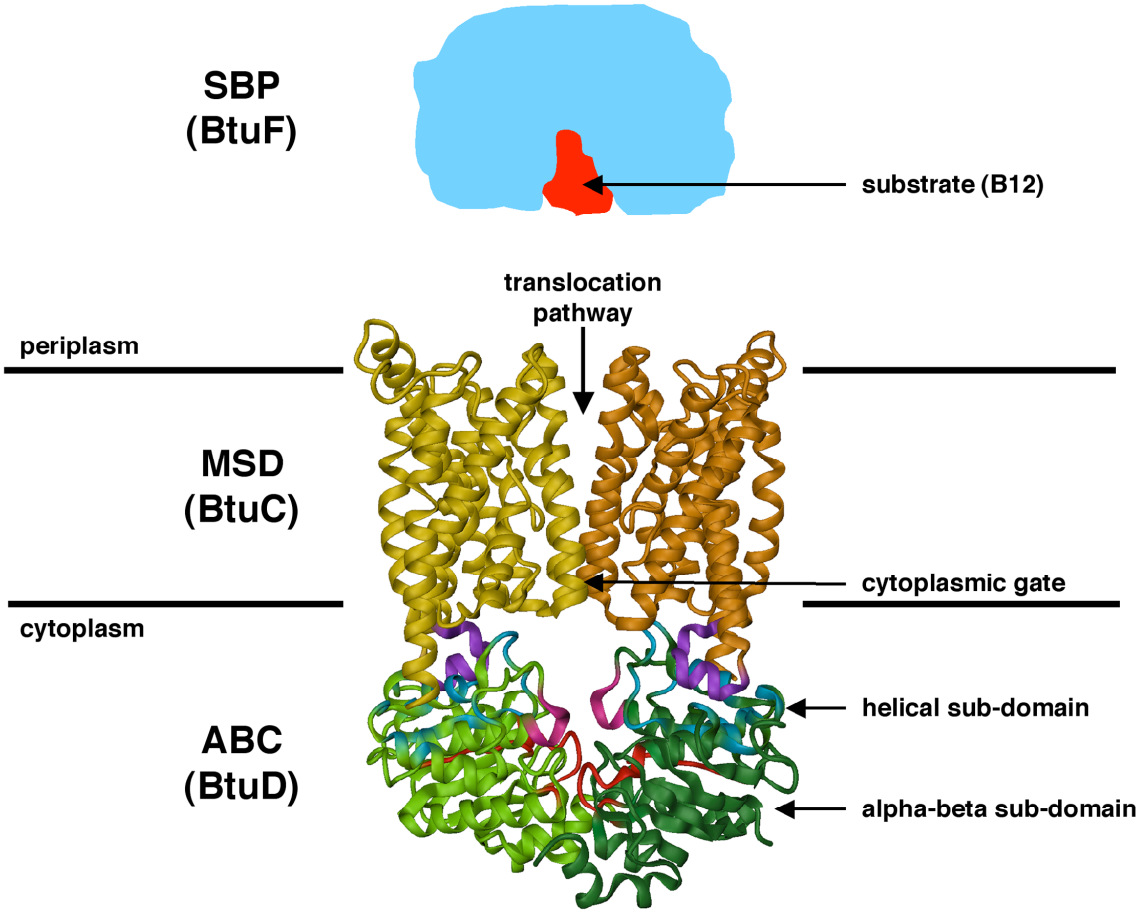

Figure 3. BtuCD-F: a bacterial SBP-dependent ABC importer for vitamin B_{12}. BtuC, BtuD and BtuF are colored as in Figure 2, with the following exceptions: the L-loop of BtuC is purple, the Q-loop and surrounding residues that contact BtuC are blue, the Walker A, Walker B and switch regions are in red, and the ABC signature motif is pink. ATP would be bound at the interface of the two ABC domains between the Walker A (red) and ABC signature (pink) motifs.

Complete transporters: exporters. The structure of one bacterial exporter, MsbA, from both *E. coli* and *V. cholera*, has also been solved (28, 29). MsbA is a member of the P-glycoprotein/MDR1 family of ABC transporters that flips lipid A out of the cytoplasmic leaflet of the membrane. The crystal structure of MsbA from *E. coli* (28) revealed a dimer of half-transporters in which the interface between MSDs is limited to the periplasmic half of the membrane. This arrangement is strikingly different from that

observed at the BtuC dimer interface. Each MsbA MSD consists of six transmembrane alpha helices packed in a bundle. Also in striking contrast to BtuCD, the ABC domains make no contact with each other and a large region including the Walker A motif is not resolved. Connecting the MSDs and ABC domains is the intracellular domain (ICD), a helical domain formed from cytoplasmic loops between MSD helices. In the structure of *V. cholera* MsbA (29), the two MSDs are significantly closer together. While being essentially conserved, there are a few secondary structural changes in the ICD. The two ABC domains in this structure are fully resolved and make contact with each other. However, the region of the ABC domain containing the Walker A motif is rotated 90 degrees from its relative position in BtuD and other ABC domains of known structure. It is currently unclear if these differences between the two MsbA structures and between MsbA and BtuCD have functional implications.

The Transport Cycle and Coupling between ATP Hydrolysis and Transport

ATP hydrolysis is required for substrate transport by all ABC transporters, presumably to energize and coordinate conformational changes within the ABCs and MSDs that permit substrate translocation (5, 7, 8). In SBP-dependent systems, the SBP may provide a second source of (binding) energy and signal the presence of ligand to the ABC domains (30). But how are the delivery of substrate by the SBP, ATP hydrolysis by the ABC domains and substrate translocation through the MSDs coupled?

Conformational changes in the ABC domains. As noted above, two molecules of ATP can be bound at the ABC dimer interface, each nucleotide contacting the Walker A motif of one subunit and the ABC signature motif of the other. It has been shown structurally and biochemically that the presence of ATP serves to draw the two ABC subunits together, tightening the dimer interface (Figure 4, part iii) (7, 12, 31-33). The structure of BtuCD was solved in the absence of ATP and illustrates a semi-open dimer interface (12). Conformational flexibility has also been noted between the alpha-beta and the helical sub-domains of isolated ABC domains. These structures suggest that the presence of ATP serves to lock the alpha-beta and helical sub-domains into a fixed relative orientation (7).

Conformational changes in the MSDs. Conformational changes in the MSDs during ATP hydrolysis and substrate translocation (Figure 4, parts ii-iii) are likely to be significant based on low resolution structural data (34) and consideration of the size of the substrates that can be transported (e.g., vitamin B_{12} (1350 Da)). The structure of BtuCD shows a closed gate on the cytoplasmic side of the membrane that presumably opens to allow B_{12} passage into the cell (12). The exact nature of any rearrangements within BtuC or other MSDs, however, waits to be elucidated in future structural and biochemical studies.

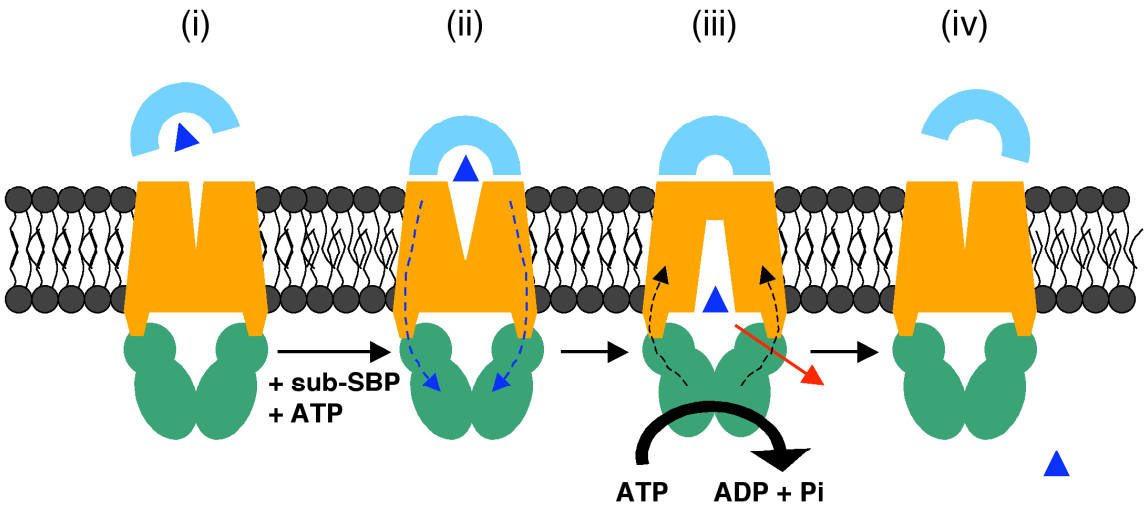

Figure 4. Model of the SBP-dependent ABC transport cycle. In stage (i), the SBP (light blue) with bound substrate (triangle) binds to the periplasmic surface of the MSDs (orange) of the transporter. In stage (ii), the bound substrate-SBP sends a signal (blue dashed arrows) to the ABC domains (green), stimulating ATP hydrolysis. In stage (iii), ATP hydrolysis by the ABC domains drives conformationl changes in the MSDs (black dashed arrows) that allows substrate translocation. In stage (iv), the substrate is now in the cytoplasm and the SBP is free to bind another substrate molecule.

The transmission interface. ATP-dependent conformational changes in the ABC domains are almost certainly transmitted to the MSDs via the transmission interface (described above) (Figures 3 and 4, part iii). Two theories have been proposed about how the tightening of the ABC dimer interface translates into opening of the cytoplasmic gate. The first, proposed by Locher et al. (12, 27) (Figure 4) is based on the crystal structure of BtuCD and says that tightening of the BtuD interface pushes out on the L-loop region of BtuC, thus forcing the gate open and allowing B_{12} passage. The second theory, proposed by Chen et al. (7, 35), is based on crystal structures of the MalK ABC domains in the presence of various nucleotides and says that tightening at the dimer interface pulls on the transmission interface, closing the cytoplasmic gate and opening the

periplasmic MSD interface. In this model, the cytoplasmic gate is open in the resting state of the transporter, thus suggesting that the structure of BtuCD solved in the absence of nucleotide actually represents a state between resting and the transition state for ATP hydrolysis. It will obviously require further structural and biochemical studies of intact transporter systems to clarify this mechanism.

The transmission interface may be an architecturally conserved feature of all ABC importers and exporters (27). Superposition of the crystal structures of BtuD and the ABC domains of CFTR and Tap1 reveals that mutations in CFTR and Tap1 affecting coupling of ATP hydrolysis and transport or the assembly of the transporter locate to the region of BtuD contacting the L-loop of BtuC. Mutant analysis and sequence alignments further reveal that the L2 helix of the L-loop may also be a conserved feature. It must be noted, however, that MSDs and ABCs do not generally "mix and match" (10). Every MSD has a cognate ABC domain and so there must also be an element of molecular recognition inherent at the transmission interface as well. If this region proves to be architecturally conserved, it will provide further evidence that the mechanism of transport is conserved across the family of ABC transporters.

The role of SBPs in the transport cycle. In the maltose (MalFGK-MBP) and histidine (HisQMP-J) SBP-dependent transport systems, both liganded and unliganded SBPs have been shown to bind to their cognate transporters and stimulate ATP hydrolysis by the cytoplasmic ABC domains (36, 20, 37). However, both histidine-bound HisJ and maltose-bound MBP stimulate to a greater extent than HisJ or MBP alone. As noted

above, MBP undergoes a large conformational change in the presence of maltose and it is believed that this change can be sensed by the MalFGK transporter, leading to increased ATP hydrolysis by MalK (20, 22). SBPs like BtuF do not open and close with substrate binding (25 and Appendix 1) and it is currently unclear if the BtuCD transporter can sense the presence of B_{12} bound to BtuF during the transport cycle (Chapter 3).

The maltose transport system has been analyzed using vanadate to trap the presumed transition state for ATP hydrolysis. These studies have revealed that MBP, which normally has somewhat low affinity for its cognate transporter, is required for vanadate trapping and remains stably bound to the transporter in this state (30, 38). Furthermore, maltose has been released and MBP appears to be in an open conformation (39). ATP hydrolysis is also required for formation of the vanadate-trapped complex, even though ADP is the nucleotide bound to MalK along with vanadate. These results suggest that substrate transport occurs before collapse of the transition state but that SBP release requires the loss of ADP and/or P_i . These results also suggest that both the SBP and ATP work to keep transport unidirectional. The SBP, tightly bound during ATP hydrolysis, may act like a gate closing the pathway back into the periplasm while the forward gate into the cytoplasm (formed by the MSDs) is opened to allow substrate translocation.

ATP-substrate stoichiometry. Estimates of the ratio of ATP molecules hydrolyzed to substrate molecules transported range from 1 to 70 (40 and Chapter 3). Measurements based on growth yields in bacteria suggest the number is only one ATP per substrate (40).

This calculation becomes quite complicated in some systems that exhibit a basal level of hydrolysis in the absence of any signal (binding protein and/or substrate) (41 and Chapter 3). It is still unclear what the physiological role, if any, of such uncoupled ATPase activity is. An *in vivo* stoichiometry of two ATPs per substrate translocated is a popular theory since there are two ATP binding sites per transporter. A recent study on the glycine-betaine ABC transporter, OpuA, reconsituted *in vitro* suggests the ratio is indeed two (42). The authors believe this estimate to be particularly reliable because of the very tight coupling observed between ATP hydrolysis, the osmotic signal and the presence of substrate.

Obviously, further functional and structural studies are required to elucidate all the conformational changes associated with transport, as well as the number of ATP molecules required per transport event and the precise place at which ATP binding, hydrolysis and release of hydrolysis products fit into the transport cycle.

Vitamin B_{12}

The structure of cobalamin. B_{12} or cobalamin is a large, complex and beautiful cofactor that consists of corrin ring with a central cobalt atom and a nucleotide loop that contains a 3' phosphoribosyl-dimethylbenzimidazole (DMB) group (Figure 5) (43). In solution, the nucleotide loop swings around allowing a nitrogen of the DMB group to act as an axial ligand to the cobalt. In some B_{12}-dependent enzymes, the nucleotide tail is bound by the protein and a histidine side chain takes its place in order to modulate the reactivity of the

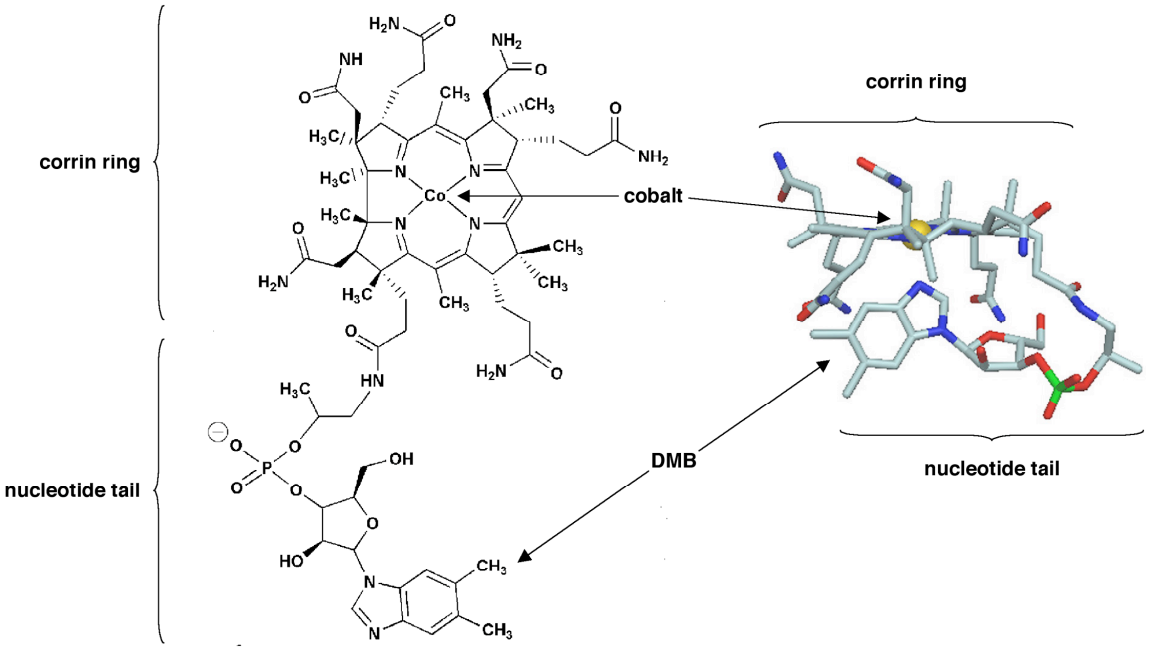

Figure 5. Vitamin B_{12}. The chemical structure and three-dimensional crystal structure of vitamin B_{12} are shown on the left and right, respectively. In the crystal structure, carbon atoms are colored grey, nitrogen blue, oxygen red, phosphorus green and the central cobalt atom is represented by a gold sphere.

cofactor. The second axial ligand to the cobalt varies. Industrial synthesis of B_{12} leaves a cyano group (CN) in this position. CN-cobalamin is also known as vitamin B_{12}. In B_{12}-dependent methyl transferases, the second axial ligand is a methyl group (Met-B_{12}), while ribonucleotide reductase requires an adenosyl group in this position (Ado-B_{12}).

The biosynthesis of cobalamin and its use by various organisms. B_{12} is thought to be very ancient in origin and may even have been synthesized and used pre-biotically in the "RNA world" (43). B_{12} is structurally and biosynthetically related to heme and chlorophyll, though analysis of their biosynthetic pathways suggest that the pathway first evolved for B_{12} and was later adapted to heme and chlorophyll production. Though

ancient, B_{12} has a very uneven distribution in nature today. It is essential to human beings and its absence causes pernicious anemia, but we cannot synthesize it. B_{12}-dependent enzymes in humans include methionine synthase, a methyl-transferase involved in recycling folate and producing methionine, and methyl malonyl CoA mutase, which is required to degrade odd-chain-length fatty acids. To date, no role for B_{12} has been discovered in plants, fungi and, not surprisingly, they cannot synthesize it. However, some species of bacteria, including *Salmonella typhimurium*, can do so. In fact, 1% of the *Salmonella* genome is dedicated to B_{12} synthesis and import. Paradoxically, mutations in cobalamin synthetic genes have no aerobic or anaerobic phenotype in wild type *Salmonella*. Wild type *E. coli* does not require B_{12} either and though it has retained part of the biosynthetic pathway in its genome, it cannot synthesize B_{12} *de novo*. The most ancient or fundamental role for B_{12} in bacteria appears to be the anaerobic fermentation of small molecules such as propanediol and glycerol and secondarily, for methyl transfer reactions. *Salmonella* species have retained B_{12}-dependent propanediol degradation while *E. coli* has not. Both *Salmonella* and *E. coli* have a B_{12}-dependent methyl transferase, MetH, which they will use preferentially. However, both also possess MetE, an enzyme that can catalyze the same reaction independent of B_{12}. If the MetE enzyme is not present or is not functional, then B_{12} is required for growth on methionine deficient media.

Cobalamin uptake in *E. coli*: the BtuB-CD-F system. Since it cannot synthesize B_{12}, *E. coli* imports this cofactor (43). B_{12} cannot pass through the membrane of the bacterium, and is too large to enter passively through outer membrane porins, and

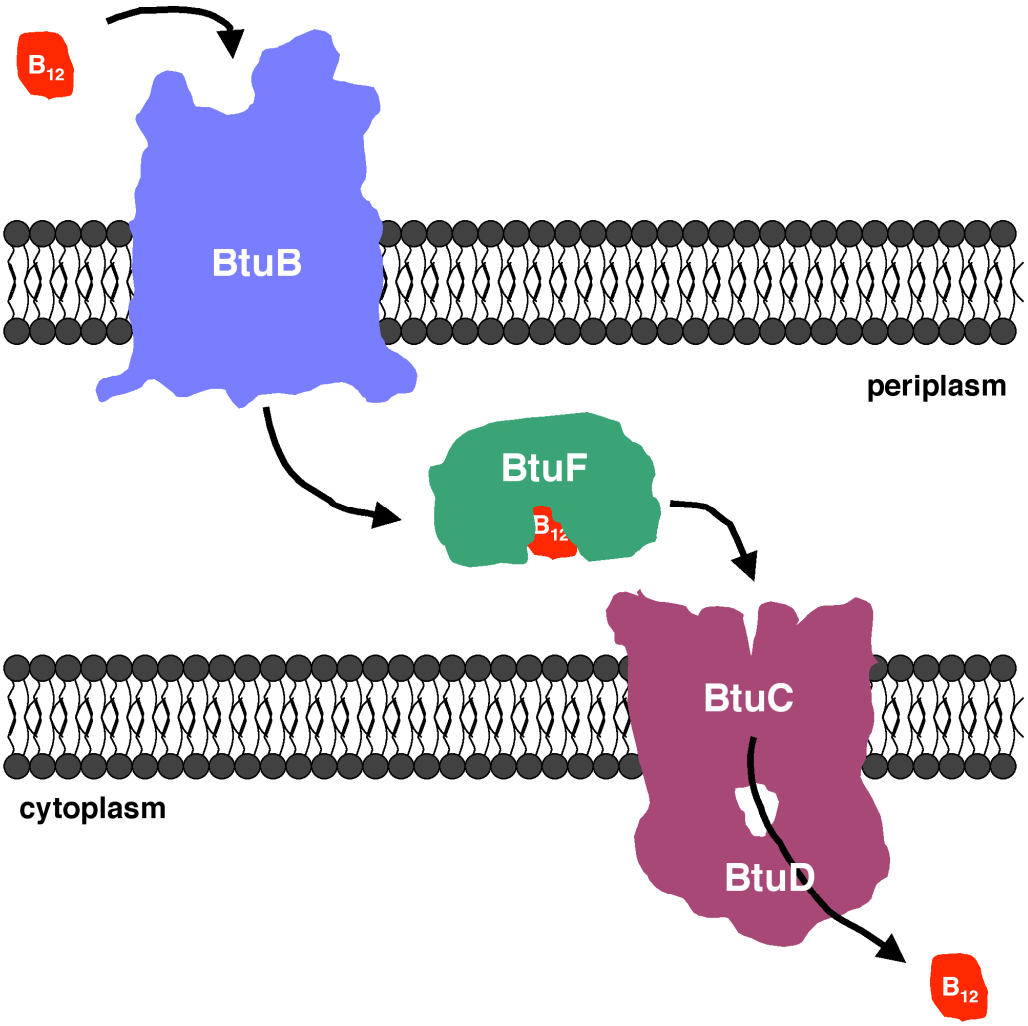

Figure 6. The Btu uptake system for B_{12} in *E. coli*. BtuB, an outer membrane integral beta-barrel protein (blue), binds B_{12} (red) in the environment with high affinity and transports it into the periplasmic space. In the periplasm, B_{12} is bound by a specific binding protein, BtuF (green), which delivers it to the inner membrane ABC transporter, BtuCD (purple). BtuCD uses the power of ATP binding and hydrolysis to transport B_{12} into the

furthermore, it may be extremely rare in the environment. Thus *E. coli* has developed the Btu uptake system (Figure 6). The first component of the system is BtuB, an outer membrane beta-barrel type transporter, that, when energized by the TonB complex, can bind B_{12} with very high affinity and accumulate it in the periplasmic space. Once in the

periplasmic space, B_{12} is bound with nanomolar affinity by its periplasmic SBP, BtuF, and delivered to BtuCD, the ABC transporter in the inner membrane. The BtuCD-F ABC transport system also appears to be a high affinity uptake system (Chapter 3).

BtuB was identified genetically in 1973 and was subsequently cloned and sequenced in 1985 (44-46). Interestingly, BtuB gene expression can be regulated by Ado-B_{12} but not by vitamin B_{12} (CN-B_{12}). A role for BtuC in vitamin B_{12} transport across the cytoplasmic membrane of *E. coli* was first elucidated in 1980 (47). Both BtuC and BtuD were cloned in 1985 (48) and their similarity to other periplasmic binding protein-dependent importers was recognized in 1986 when their sequences were determined (49). There was some confusion, however, because the periplasmic binding protein was missing from the operon containing *btuC* and *btuD*. The *btuE* gene, which resides between *btuC* and *btuD*, has no role in B_{12} uptake (50) while *btuF*, the gene for the true B_{12} binding protein, resides in a completely different part of the genome. *BtuF* was finally identified in *S. typhimurium* in 1999 (51). The analogous gene in *E. coli* was identified in 2002 (52) and the BtuF protein was purified and determined to have a K_d of 15 nM for binding to B_{12}. There is currently no evidence for regulation of BtuC, BtuD or BtuF expression levels.

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