Conformational Transitions Induced by the Binding of MgATP to the Vitamin B$_{12}$ ATP-binding Cassette (ABC) Transporter BtuCD$^*$

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ATP-binding cassette transporters use the free energy of ATP hydrolysis to transport structurally diverse molecules across prokaryotic and eukaryotic membranes. Computer simulation studies of the “real-time” dynamics of the ATP binding process in BtuCD, the vitamin B$_{12}$ importer from Escherichia coli, demonstrate that the docking of ATP to the catalytic pockets progressively draws the two cytoplasmic nucleotide-binding cassettes toward each other. Movement of the cassettes into closer opposition in turn induces conformational re-arrangement of α-helices in the transmembrane domain. The shape of the translocation pathway consequently changes in a manner that could aid the vectorial movement of vitamin B$_{12}$. These results suggest that ATP binding may indeed represent the power stroke in the catalytic mechanism. Moreover, occlusion of ATP at one catalytic site is mechanically coupled to opening of the nucleotide-binding pocket at the second site. We propose that this asymmetry in nucleotide binding behavior at the two catalytic pockets may form the structural basis by which the transporter is able to alternate ATP hydrolysis from one site to the other.

ATP-binding cassette (ABC)$^*$ transporters are a large group of proteins that facilitate the permeation of solutes across cell membranes. The energy for this process is provided by the hydrolysis of ATP (1). Members of the family so far identified include importers, exporters, receptors, and channels. Defects in a number of ABC transporters have been associated with serious hereditary disorders, of which cystic fibrosis is the best known (2). In addition, increased expression levels of certain members of this ubiquitous family are a common cause of bacterial resistance to antibiotics and the resistance of human tumor cells to anticancer drugs (3, 4). Recent successes in the determination of high resolution structures (5–16) have made it possible to use computer modeling techniques to study ABC transporters (17–21). Here, we present extensive molecular dynamics (22) simulation studies of the ATP binding process in Escherichia coli BtuCD, an integral inner membrane protein that mediates the import of vitamin B$_{12}$. Although ATP-driven dimerization of the ATP-binding cassettes has been predicted to represent the power stroke in the catalytic cycle (13), the crystal structure of an intact dimer (including the transmembrane segments) in the ATP-bound state is unfortunately yet to be reported. Two computer simulations were designed to investigate the hypothesis that the transition from a weak to a tight ATP binding state may elicit the conformational changes needed to unidirectionally force vitamin B$_{12}$ through the transmembrane pathway.

The biologically functional unit of ABC transporters is typically dimeric. It consists of two transmembrane domains (TMDs) that function as a pathway for the permeation of solute and two cytoplasmic nucleotide-binding domains (NBDs). The highly conserved NBDs are the nucleotide-hydrolyzing engines that drive transport through the TMDs (see Fig. 1). Conserved segments found in the NBD include the Walker A motif (or P-loop), the Walker B motif (23), the Q-loop, the D-loop, the H-motif, and the LSGGQ sequence. The Walker A and B motifs are found in other ATP-binding proteins as well, whereas the LSGGQ segment is the signature motif of ABC transporters (24).

The crystal structure of the NBD of a histidine importer, HisP (5), showed that each monomer has an overall “L” shape. The Walker A and B motifs are located in arm I, whereas the signature motif is in arm II. Other structures of NBDs that have since been solved include the structure of Rad50, an ATPase involved in DNA repair, which revealed the formation of a head-to-tail dimer in the presence of bound ATP but without the Mg$^{2+}$ cofactor needed for hydrolysis (15). The nucleotide was sandwiched between the Walker A and B motifs of one NBD and the signature motif of the opposing NBD. This had been predicted earlier through modeling studies by Jones and George (25). Recent structures of dimeric MJ0796 and MalK have shown that the head-to-tail sandwich dimer can exist in true ABC transporters (9, 26). In the case of MJ0796, the dimer was obtained only after an E171Q mutation of the highly conserved catalytic glutamate at the end of the Walker B motif. The fact that the E171Q mutant is defective in ATP hydrolysis is believed to have stabilized the dimeric form. This was supported by studies on the glucose importer GloV from Sulfolobus solfataricus (15). The NBD dimer of MalK, on the other hand, was obtained from the wild-type protein. Nevertheless, ATP hydrolysis was prevented by excluding Mg$^{2+}$ using EDTA. It is also notable that MalK is different from MJ0796 and several other ABC transporters in that it has an additional regulatory domain, which may stabilize the nucleotide-bound dimer. Unlike the strongly conserved NBDs, the TMDs show significant variation. This divergence may account for the diverse range of molecules translocated by members of ABC transporter family. Known transport substrates vary from anions, lipids, and amino acids to peptides, polysaccharides, and whole proteins (24).
Three structures of complete transporters have been reported: the lipid A exporter MsbA from E. coli (Eco-MsbA) (8) and Vibrio cholera (VC-MsbA) (7) and the E. coli vitamin B12 importer BtuCD (11). Although all three had no nucleotides bound at their catalytic sites, the structures were significantly different in conformation. In the Eco-MsbA structure, the NBDs are ~50 Å apart with the nucleotide-binding sites facing away from the dimer axis. In VC-MsbA and BtuCD, they are in close opposition, facing each other in the same way as Rad50. However, the orientation of arm I of the NBD of VC-MsbA relative to arm II differs from that of BtuCD. A conformational arrangement similar to that of VC-MsbA has not been reported in previously solved structures of isolated NBDs with or without nucleotide. These variations may be indicative of the ability of the NBD to sample a large conformational space. Alternatively, they could be indicative of the crystal packing forces in MsbA.

Although the rapidly growing body of structural data has helped to clarify the nature of subunit-subunit and nucleotide-protein interactions, details regarding the mechanism by which binding, hydrolysis, and release of nucleotide are coupled to steps in the transport cycle remain unresolved or controversial. We have simulated the transition of the NBDs of BtuCD from a semi-open state, as observed in the crystal structure, to a MgATP-bound closed state. Our simulations show that concomitant conformational transitions take place in the transmembrane domain, leading to the closure of the periplasmic end of the transportation pathway and initiating the opening of the cytoplasmic gate of the pathway. Although a similar mechanistic model was suggested for BtuCD by Locher et al. (11), it has recently been disputed by Chen et al. (9) based on their results on the ATP-bound dimer of the maltose transporter, MalK. They have instead proposed a tweezer-like model in which nucleotide binding leads to the reverse effect, i.e., closure of the cytoplasmic gate and opening of the periplasmic end. Our findings on conformational changes associated with MgATP binding to BtuCD are likely representative of bacterial importers in general and may be extensible to other ABC transporters.

MATERIALS AND METHODS

Starting coordinates for the simulations were obtained from the x-ray structure of BtuCD (Protein Data Bank entry 1L7V) (11). Cycloextravatanadate was located in the ATP-binding sites in the crystal structure but did not affect the conformation of the NBD (11). We docked a MgATP molecule at the Walker A and B motifs of each of the two NBDs for the first simulation by superimposing the monomeric ATP-bound crystal structure of the nucleotide-binding domain of HisP (5) onto the equivalent domains of BtuCD. A preequilibrated bilayer of 288 palmitoyloleoylphosphatidylethanolamine lipids solvated in single point charge water (28) was used as a model of the biological membrane. Lipids were removed to generate a hole in the center of the bilayer. In a short simulation using the procedure of Faraldo-Gomez et al. (29), remaining lipids were removed from the hole to generate a BtuCD-shaped cavity. The protein was inserted into the cavity. The entire system was then resolvated, and 18 chloride counter ions were added. The system (98,084 atoms) was equilibrated for 250 ps with positional restraints applied on the protein atoms (Fig. 1) to allow the solvent to relax. A second independent simulation without MgATP was set up from the same protein and lipid coordinates, (nucleotide-free) with Fig. 2, to allow the solvent to relax. A second independent simulation without MgATP was set up from the same protein and lipid coordinates, (nucleotide-free) with Fig. 2, to allow the solvent to relax. A second independent simulation without MgATP was set up from the same protein and lipid coordinates, (nucleotide-free) with Fig. 2, to allow the solvent to relax. A second independent simulation without MgATP was set up from the same protein and lipid coordinates, (nucleotide-free) with Fig. 2, to allow the solvent to relax.
simulation. Indeed, the separation distance between the centers of mass of the P-loop and the signature motif in the opposing NBD decreases by 0.4 Å at the catalytic site shown in Fig. 2C. The equivalent distance increases by 1.6 Å at the alternate site, shown in Fig. 2B. In contrast, the corresponding distances in the nucleotide-free simulation both increase by 2.2 and 2.6 Å, respectively (Fig. 2A).

Events at the cytoplasmic end of the transmembrane pathway were analyzed by measuring the distance between the centers of mass of Thr-142 and Ser-143 located at the turn between membrane-spanning helices TM4 and TM5 in each half of the transporter. These residues were identified in the crystal structure as defining the gate region leading from the transmembrane pathway into the cytoplasmic water-filled channel at the interface of the NBDs and the TMDs (11). As the NBDs are drawn closer to each other due to ATP binding, the size of the gate region opening increases. This suggests that tighter ATP-induced association of the NBDs and the change in gate aperture are coupled. Analysis of the evolution of hydrogen-bonding patterns between ATP and the binding pocket and between the two NBDs suggests that closer association of the NBDs is aided by the formation of hydrogen bond contacts between residues of the two ATP-binding cassettes.

**ATP Binding**—Over a time span of only 4 ns, significant conformational changes occur to optimize ATP binding at one of the two active sites in the ABC dimer. Surrounding water molecules were progressively displaced from hydrogen-bonding sites within the nucleotide-binding pocket located at the interface of the two opposing ATP-binding cassettes (Fig. 3, A and B). The signature motif in the opposite subunit reached out toward the docked ATP molecule. The nucleotide also shifted toward the signature motif and reoriented itself to form stable hydrogen bonds between the two side chain oxygen atoms of the glutamate residue in the LSGGE signature motif and the two hydroxyl hydrogen atoms in the ribose moiety of ATP. This is consistent with equivalent hydrogen bonds formed between the ribose hydroxyls and the corresponding Gln of the LSGGQ motifs in the ATP-bound dimers of MalK and E171Q-MJ0796 (26). The Glu residue of the LSGGE signature motif of BtuCD also forms a water-mediated hydrogen bond with one of the γ-phosphate oxygen atoms of ATP. Coordination of the magnesium ion cofactor occurred via the α-, β-, and γ-phosphate oxygen atoms of ATP, Gln-80 of the Q-loop, as well as Ser-40 of the Walker A motif and a solvent water molecule. Direct contact between metal ion cofactors and Gln-80 has been similarly observed in the dimeric crystal structures of Rad50-AMPPNP-Mg$^{2+}$ and E171Q-MJ0796-Na$^+$ (13, 26).

**Allosteric Control of Translocation Pathway**—The transmembrane translocation pathway becomes constricted as the crystal structure relaxes to new conformations in solution without bound MgATP (Fig. 4, A and B). It is, however, evident from a comparison of the MgATP-bound and MgATP-free structures at 15 ns (Fig. 4, B and C) that further constriction of the translocation pathway occurs as an effect of nucleotide occupancy. Fig. 4, A–C, show that the reorientation of transmembrane helix 5 (TM5) in each monomeric unit plays an important role in mediating changes in pathway profile.

To quantitatively map out the changes taking place in the membrane-spanning pathway, separation distances between the equivalent Cα atoms of TM5 from each subunit were measured at approximately every turn of the helix from the cytoplasmic...
to the periplasmic end. The results are presented in Fig. 5. In the crystal structure, the periplasmic end of the translocation pathway was open wide enough to accommodate a vitamin B12 molecule (11). Our results show that the periplasmic opening collapses by \( \approx 7 \) Å in the ligand-free simulations. This implies that the periplasmic entrance may be more constricted in solution than it is in the crystal structure. The observed mobility may also explain why residues at the entrance to the periplasm were only partly resolved in the crystal structure (11). Fig. 5 shows that the docking of MgATP at the catalytic sites allosterically induces further constriction of the translocation pathway by up to 5 Å. Profile differences of interest are also observable at the gate region (Thr-142 and Ser-143) that leads into the water-filled channel at the center of the transporter. Vitamin B12 is believed to escape into the cytoplasm of the cell through this channel. The dimension of the opening at the gate region increases in response to the docking of MgATP, whereas in the absence of MgATP, it becomes slightly narrower, as evident in the segment containing residues 142–150 in Fig. 5.

DISCUSSION

High resolution structures have played an important role in aiding our understanding of the mechanism of ABC transporters. Protein function, however, involves movement, and molecular dynamics simulations are one way of probing potential functional motions starting from rigid crystal structures. Several recent computer simulation studies have demonstrated the usefulness of accessible conformational states in unraveling the functional mechanics of protein systems comparable with BtuCD (22, 37, 38). The main limitation of modern simulations is the fact that conformations separated by high energy barriers are unlikely to be visited during the simulation on a time scale that can be simulated directly. The fast changes observed in the nucleotide-binding domains and in the transmembrane domains indicate significant driving forces for these motions.

Nucleotide Binding—Nucleotide binding is a two-step process. First, MgATP diffuses and docks into the catalytic pocket, producing a weak binding conformation of the complex. The nucleotide is subsequently sequestered, and binding interactions are optimized to generate a tight binding conformation. For F₁F₀-ATP synthase, Antes et al. (39) have suggested that energy transduction occurs during the transition from the weak to the tight binding state. In our simulation, we circumvent the slow diffusion process and start with the MgATP weakly docked at the binding pocket. Within only 4 ns, there is evidence that the transition of the complex from the weak to the tight binding state has largely occurred. The resulting dimer likely represents the catalytically competent state of the ATP-binding cassette (13). The short time scale in which this transition takes place is an indication of the strong attractive force involved and suggests that the energy transduced as a result may be sufficient to drive the power stroke of the transporter. However, we observe that just one ATP molecule appears capable of holding the transporter in the closed nucleotide-bound state. Tight ATP binding at one catalytic site occurs concomitantly with opening of the binding pocket at the alternate site in a manner that may help create an exit path for the release of hydrolyzed nucleotide. This finding may at first
appear contrary to evidence from recent crystal structures of dimeric NBDs of Rad50, MJ0796, and MalK in which ATP is bound at each of the two catalytic sites (9, 12, 13). However, it is notable that in all three cases either the protein used was a catalytically incapacitated mutant (MJ0796 (E171Q)) or the magnesium cofactor was excluded to prevent hydrolysis (Rad50 and Malk). The crystal structure of catalytically competent wild-type NBDs with MgATP stably bound at both nucleotide-binding sites has so far proven elusive. We suggest that this may be attributed to the asymmetry in ATP binding observed in our simulations. The singly occupied dimer is unstable without additional contacts provided by the transmembrane domain. Under non-hydrolyzing conditions, the two binding sites are more likely to be simultaneously occupied with ATP. Together, both ATP molecules may thus provide sufficient contacts to stabilize the dimer in the absence of transmembrane domains as observed in the crystal structures of Rad50, MJ0796, and MalK. Other lines of experimental evidence in support of this kind of asymmetry include vanadate trapping studies in which the protein is incubated with vanadate and [α-32P]MgATP (40). A trapping ratio of 1 mol of nucleotide/mol of transporter has been demonstrated for both P-glycoprotein and the maltose importer (41, 42). This ratio and the fact that vanadate trapping is assayed by analyzing the radioactivity of nucleotide (not vanadate) indicate that not only is MgADP-vanadate trapped at a single catalytic site but also that the second site is not occupied by ATP or ADP. In this context, the observation in our simulation that binding at catalytic site 1 is tightly coupled to the opening of the binding pocket at site 2 has significant implications because it rationalizes a number of presently unresolved issues regarding the catalytic mechanism. In P-glycoprotein, an alternating site hydrolysis mechanism has been proposed based on the observation that vanadate-induced trapping of one site in the catalytic transition state prevents the second site from turning over (40). It has, however, been difficult to perceive how this alternation could occur within the framework of a symmetric dimer containing two bound ATP molecules. How, for example, would the dimer interface be broken to allow release of hydrolyzed nucleotide while still retaining the “recollected” to effect hydrolysis at the alternate site upon reconstitution of the catalytically competent dimer? Besides, this sequence would need to be repeated in a concerted fashion for the pumping mechanism to be efficient. Our model does suggest a mechanical basis by which the transporter keeps the “conformational memory” needed to alternate ATP hydrolysis between the two catalytic sites. In the presence of the transmembrane domains, the two NBDs do not have to dissociate completely during the catalytic cycle. The binding pocket could conceivably open to release the products of nucleotide hydrolysis at one site while maintaining the dimer interface at the alternate site, where the subsequent ATP hydrolysis reaction occurs. This prevents wastage of the binding energy of ATP at the second site, which would be expected to be the case in a mechanistic model that involves the dissociation of a doubly occupied ATP sandwich dimer. Since only one ATP molecule can apparently hold the dimer in the catalytically competent state, nucleotide triphosphate may be recruited and nucleotide diphosphate may be released as the pump fires alternately from each of the two catalytic sites in a manner reminiscent of the two-cylinder engine model proposed for LmrA (43). The resulting continuous transduction of energy would allow for the high turnover rate needed for efficiency in the transport process.

Furthermore, there is substantial experimental evidence indicating that the two nucleotide-binding sites work alternately in a strongly cooperative fashion. However, the nature of the allosteric communication that allows this kind of cooperativity is still unclear. The coupling of ATP binding at one site to ATP hydrolysis and MgADP release at the alternate site as suggested by these computer simulation results may represent the basis of cooperativity. In view of experimental results suggesting that the release of nucleotide diphosphate is the rate-limiting step in the catalytic cycle (44, 45), the observation that the binding of nucleotide at one site might promote product dissociation at the second site may be an important adaptation for accelerating the release of MgADP. Comparable results have been reported for F1F0-ATP synthase in which ligand binding to two of the nucleotide-binding sites in the (αβ3)_3 subunit was proposed to provide the driving force required to open the third and thus facilitate the release of product (46). The validity of the foregoing proposed aspect of the catalytic mechanism in ABC transporters could be probed using fluorescence energy transfer experiments to estimate distances between donor and acceptor pairs covalently linked to substituted cysteines near the Walker A and signature motifs at each nucleotide-binding pocket.

Conformational Changes Induced by Nucleotide Binding— ATP binding and hydrolysis have been associated with significant conformational transitions in human MDR1 (47–49). Our computer simulation results suggest this might be the case for BtuCD and possibly other bacterial ABC transporters as well. The tendency of the periplasmic end of the translocation pathway to collapse with or without ATP indicates that the substrate-binding protein (BtuF) opens the periplasmic end of the translocation pathway upon docking onto BtuCD. It is thus likely that in its relaxed non-functional state, the periplasmic opening into the pathway is diminished in size. Docking analysis suggested that the periplasmic binding protein BtuF can be docked on the BtuCD structure (50). The conformation of BtuF, however, did not change much between the apo and holo forms (51). For the maltose import system, the periplasmic substrate-binding protein is required to associate tightly with the transporter to enable the transfer of substrate to the translocation pathway (41). From these results, we infer that BtuC undergoes conformational rearrangement to align to and thus dock correctly with liganded BtuF. The mobility of the pathway entrance in the absence of BtuF may thus be a reason why residues lining the periplasmic entrance to the translocation pathway were only partly resolved in the BtuCD crystal structure (11). We further propose that the tight interaction between BtuCD and liganded BtuF is disrupted when ATP-induced dimerization of the NBDs forcefully drives closure of the periplasmic entrance. The resulting constriction vectorially pushes the vitamin B12 molecule from the entrance toward the cytoplasm. A noticeable cavity is maintained inside the transmembrane pathway in our simulations, implying that vitamin B12 is possibly sequestered into a microenvironment that favors its motion inward through the pore. Such a scenario would be consistent with the peristaltic motion mechanism suggested by Locher et al. (11). It is possible that the force transduced in this way may be sufficient to completely push the vitamin B12 molecule into the cytoplasm. A simulation incorporating the periplasmic substrate-binding protein BtuF and vitamin B12 would provide useful clues in this regard. Analogy to predictions for MsBA suggests that interaction between the wall of the pore and vitamin B12 may play a role in adding impetus to the transportation process (8). The significant changes in the shape of the translocation pathway presented in Figs. 4 and 5 imply that ATP-induced dimerization drives most of the translocation process. Further energy transduction due to hydrolysis may serve the purpose of finally ejecting vitamin B12 into cytoplasmic space. Calculations involving F1F0-ATP synthase...
show that cleavage of the bond between the β and γ phosphates of ATP in the βDP catalytic site produce a relatively small change in free energy (ΔG = −8.9 kcal/mol) (52). Proposed Mechanism—Incorporating our findings on the “real-time” dynamics of BtuCD into the existing body of experimental data and building on concepts from previous models, we posit that a simplified general mechanism of the catalytic cycle of bacterial ABC importers may proceed following the sequence outlined in Fig. 6. In step 1 of the proposed mechanism, BtuF preloaded with vitamin B12 docks onto BtuCD and causes BtuC to undergo a conformational change that results in the opening of the periplasmic entrance into the translocation pathway as inferred from our simulation study. In the nucleotide-biding domain, a weakly docked MgATP molecule occupies catalytic site 1 (top pentagon), whereas site 2 has a newly hydrolyzed nucleotide molecule (bottom square) consistent with the principle that hydrolysis alternates between the two sites (40). It is known in the maltose transport system that cleavage of the bond between the elements that the binding of just one molecule of MgATP causes significant changes in the translocation pathway. Transition to a tight MgATP binding state at site 1 induces closer association of the two NBDs and promotes the release of MgADP at site 2. This causes a reorientation of helices in the TMDs, leading to shuttling of the substrate molecule through the translocation pathway. Based on our results, we speculate that closure of the periplasmic entrance induced by MgATP binding causes contacts between BtuF and BtuC to be broken so that BtuF is dislodged into the periplasm. This MgATP-driven release of BtuF may be necessary in view of the fact that importers have been reported to form tight complexes with the periplasmic binding protein (50). Moreover, step 2 is in agreement with data suggesting that it is nucleotide binding rather than ATP hydrolysis that drives the huge conformational shift required to drive solute translocation in P-glycoprotein (47, 49). In step 3, the hydrolysis of MgATP at site 1 following the release of vitamin B12 from the transporter resets the transmembrane domains and completes one transport event. Sharom and coworkers (54) have shown that the release of drug from P-glycoprotein during the catalytic cycle precedes the formation of the vanadate-trapped transition state. Similar results have also been obtained in the maltose transport system, in which maltose was found to be absent from the vanadate-trapped transition state complex (41). It has also been reported that hydrolysis can occur at site 1 even if site 2 is not intact (55). At the end of step 3, the transmembrane domains are ready to accept another liganded BtuF complex, whereas nucleotide-binding site 2 is also available to accept another MgATP molecule. Step 4 is a repeat of step 1. BtuF docks onto BtuC again and releases a new molecule of vitamin B12. Step 5 is a repeat of step 2 except that the power stroke is now driven by NBD dimerization induced by tighter ATP binding at site 2 rather than site 1, in line with the alternating site mechanism. In step 6, a full cycle of the mechanism is completed as hydrolysis occurs at site 2. Nikaido and Ames (55) have proposed a similar model in which ATP hydrolysis and the exit of inorganic phosphate lead to the release of solute from the periplasmic binding protein and its subsequent transportation through the transmembrane pathway. In our model, nucleotide binding and subsequent hydrolysis at one site would be sufficient to complete the translocation of one molecule of vitamin B12, giving a 1:1 stoichiometry of one molecule of ATP/imported vitamin B12 molecule. The mechanistically crucial issue of stoichiometry has not been fully clarified yet. Experiments monitoring bacterial growth suggest a 1:1 ratio, whereas a recent study of the OpuA transporter strongly suggested that two ATP molecules are needed for each transport event (56, 57). Our simulations do not provide enough information to obtain the stoichiometry and models based on a 2:1 ratio of ATP to vitamin B12 would be possible.

In conclusion, the results of this study show that MgATP binds asymmetrically to the two catalytic sites in the NBDs of BtuCD. The occlusion of nucleotide at one binding site is accompanied by increased exposure of the second site to the solvent. The binding of MgATP can also be linked to structural transitions in the transmembrane domain of the transporter. Conformational changes induced by nucleotide binding are discussed in the context of their possible roles in the mechanism by which vitamin B12 is imported into bacterial cells. In particular, alternation in ATP hydrolysis between the two catalytic sites is likely to be facilitated by the observed asymmetry in nucleotide binding.

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