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Nucleotide Binding Domain Interactions During the Mechanochemical Reaction Cycle of ATP-Binding Cassette Transporters

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ATP-binding cassette (ABC) transporters serve as importers and exporters for a wide variety of solutes in both prokaryotes and eukaryotes, and are implicated in microbial drug resistance and a number of significant human genetic disorders. Initial crystal structures of the soluble nucleotide binding domains (NBDs) of ABC transporters, while a significant step towards understanding the coupling of ATP binding and hydrolysis to transport, presented researchers with important questions surrounding the role of the signature sequence residues, the composition of the nucleotide binding sites, and the mode of NBD dimerization during the transport reaction cycle. Recent studies have begun to address these concerns. This mini-review summarizes the biochemical and structural characterizations of two archaebacterial NBDs from *Methanocaldococcus jannaschii*, MJ0796 and MJ1267, and offers current perspectives on the functional mechanism of ABC transporters.

KEY WORDS: ABC transporter; NBD; dimerization; MJ0796; MJ1267; catalytic carboxylate; nucleotide binding.

INTRODUCTION

Adenosine triphosphate (ATP)-binding cassette (ABC) transporters couple nucleotide binding and hydrolysis to the transport of a wide variety of solutes across biological membranes. This gene superfamily is prevalent throughout the three domains of life, accounting for, on average, 3 and 5% respectively of each archaebacterial and eubacterial genome sequenced to date (Ren *et al.*, 2004), while eukaryotic ABC transporters range in number from the 31 found in *Saccharomyces cerevisiae* to 48 human transporters to over 100 found in *Arabidopsis* and *Oryza* (Bauer *et al.*, 1999; Dean *et al.*, 2001; Garcia *et al.*, 2004). Over one third (17 of the 48) of the human ABC transporters have been linked to genetic disorders (Dean and Annilo, 2005), the most common of them being cystic fibrosis in which the ABCC7 gene product (cystic fibrosis transmembrane counductance regulator, or CFTR) exhibits a severely reduced, or lack of, chloride channel function. In addition to causing a variety of human diseases, ABC transporters engender multiple drug resistance in both prokaryotic and eukaryotic cells, a significant hurdle faced in the battles against microbial infection and cancer. The manner in which these proteins harness the energy of ATP binding and hydrolysis to the translocation of solutes (ranging from ions, amino acids, and sugars to large bacterial toxins and proteases) across lipid bilayers has thus garnered great interest.

ABC transporters share a common domain organization of two transmembrane domains (TMDs), comprising 6–11 α-helices spanning the lipid bilayer, and two soluble nucleotide binding domains (NBDs). Bacterial transporters are commonly encoded by separate genes specific for each TMD and NBD, with some "half" transporters containing the TMD fused with an NBD in a single gene product. On the other hand, eukaryotic ABC transporters exist either as "half" transporters (TMD–NBD) or "full" transporters, which contain all four domains (typically arranged TMD1–NBD1–TMD2–NBD2) on a single

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polypeptide. The TMDs are highly divergent, as it is these domains which likely contain the binding sites for each transporter's specific solutes. In stark contrast, NBD sequences from all levels of life are highly conserved, especially with regard to the Walker A (GxxGxGKS/T, where x can be various amino acids) and Walker B (hhhhDEP, where h is a hydrophobic residue) nucleotide binding motifs (Walker *et al*., 1982) and the ABC "signature" sequence of LSGGQ. The high degree of similarity between NBDs of all ABC transporters suggests that the mechanism of energy utilization by these systems is likewise conserved.

ABC TRANSPORTER NUCLEOTIDE BINDING DOMAIN STRUCTURES

Recent structural studies of ABC transporter NBDs have provided a glimpse at the atomic level of the specific residues involved in ATP binding and hydrolysis, complementing the genetic and biochemical data already available in the literature (reviewed in Schneider and Hunke, 1998). The crystal structure of *Salmonella thyphimurium* HisP in complex with ATP (Hung *et al*., 1998) for the first time illustrated the soluble domain's organization into a F1-type mixed α/β nucleotide binding core subdomain containing the Walker A and Walker B motifs in addition to an anti-parallel β-sheet subdomain and an α-helical subdomain containing the signature LSGGQ motif. A breakthrough in the field, this initial ABC transporter NBD structure also begged a few important questions. First of all, why were the signature sequence residues, so highly conserved throughout evolution and implicated in ATP binding and hydrolysis by mutational studies (Shyamala *et al*., 1991), located over 20Å away from the observed nucleotide binding site? In addition, why was the bound nucleotide so uncharacteristically solvent exposed? Finally, how could the dimeric arrangement of HisP molecules in the crystal explain the positive cooperativity measured in ATP hydrolysis (Liu *et al*., 1997)? These questions were addressed by subsequent studies that structurally and biochemically characterized two ABC transporter nucleotide binding domains, MJ0796 and MJ1267, from the hyperthermophilic archaebacteria *Methanocaldococcus jannaschii*.

The first two ABC transporter NBD crystal structures, along with the structure of the distantly related ABC ATPase Rad50, presented three distinct crystallographic dimers (Diederichs *et al*., 2000; Hopfner *et al*., 2000; Hung *et al*., 1998). As mentioned above, the HisP structure places the signature sequence residues over 20 Å away from the ATP molecules at the binding site, and the dimeric interface involves β-strands from the anti-parallel β-sheet subdomain (Hung *et al*., 1998). The second ABC transporter NBD structure published, that of MalK from *Thermococcus litoralis*, exhibited a different dimeric interface involving all three subdomains, although the signature sequence residues remained 20 Å distant from the pyrophosphate moieties (the electron density was not clear for the adenosine groups of ADP) (Diederichs *et al*., 2000). The crystal structure of *Pyrococcus furiosus* Rad50cd, a structural maintenance of chromosomes (SMC) family member bearing sequence similarity to ABC transporter NBDs (Hopfner *et al*., 2000) ultimately pointed the field in the right direction regarding the disputed dimeric interface.

Rad50cd, comprising an N-terminal protein fragment containing a Walker A motif and a C-terminal protein fragment containing both the Walker B motif and signature sequence, was crystallized in monomeric form in the absence of nucleotide as well as an AMP–PNP bound dimeric form (Hopfner *et al*., 2000). The dimeric structure possesses two nucleotide binding sites, located at the monomer–monomer interface, composed of residues from the Walker A and Walker B motifs from one Rad50cd and residues from the signature sequence of the opposing Rad50cd, while the anti-parallel β-sheet subdomains exist on the periphery. This preliminarily addressed the three major concerns stemming from the proposed HisP and MalK dimers, namely that the highly conserved signature sequence residues contribute to the nucleotide binding site, which is completely buried, and that two nucleotides bind at the dimer interface. The authors' suggested unified molecular mechanism for ABC transporters and SMC proteins (Hopfner *et al*., 2000) is similar to earlier predictions (Jones and George, 1999) although the Rad50cd dimeric arrangement remained to be seen in an ABC transporter.

STRUCTURAL AND BIOCHEMICAL CHARACTERIZATION OF OLIGOMERIC NUCLEOTIDE BINDING DOMAINS

Several hypotheses for the physiological ABC transporter dimer had been proposed on the basis of crystallographic dimers of HisP, Rad50cd, and MalK (Diederichs *et al*., 2000; Hopfner *et al*., 2000; Hung *et al*., 1998). Studies of the *Methanocaldococcus jannaschii* NBDs MJ1267 and MJ0796 (Karpowich *et al*., 2001; Yuan *et al*., 2001) presented evidence that strongly supported the Rad50cd model of dimerization based on the mapping of previously described mutations affecting transport in the maltose transport system and sequence conservation data onto the MJ0796 dimer model (Thomas and Hunt, 2001). In addi-

tion, the MJ1267 structural study, the first to illustrate both ADP-bound and nucleotide-free forms of the same NBD, detailed the 15° rigid body rotation of the α-helical subdomain with respect to the F1-type core and anti-parallel β-sheet subdomains in the absence of the γ-phosphate of ATP (Karpowich *et al.*, 2001). This rotation, also exhibited by the ADP-bound structure of MJ0796, was proposed to serve a role in the coupling of nucleotide binding and hydrolysis to transport.

To test the Rad50cd dimer, the near-invariant glutamate residue at the C-terminus of the Walker B motif (hhhhDE̲P) was targeted for mutagenesis (Moody *et al.*, 2002). This likely catalytic carboxylate (Yoshida and Amano, 1995) was mutated to glutamine or alanine in both MJ0796 and MJ1267. MJ0796, which exhibits a wild-type ATPase activity V_{max} of 0.2 s^{-1}, a K_m of 50 μM, and Hill coefficient of 1.7 indicating positive cooperativity, loses all detectable activity when this glutamate (E171) is mutated to either glutamine (E171Q) or alanine (E171A). Corresponding mutations (E179A and E179Q) in MJ1267 have the same effect (Moody *et al.*, 2002, unpublished results). These results suggested that the highly conserved glutamate residue indeed serves as the catalytic base in the NBDs.

Provided that their nucleotide binding capabilities are not severely affected by mutating this catalytic glutamate residue, the mutant MJ0796 and MJ1267 NBDs would be predicted to form ATP-bound homodimers based on the Rad50cd model of NBD dimerization. The ability of wild-type and E171Q MJ0796 to dimerize in the presence of ATP and ADP was measured using analytical gel filtration. Both wild-type and mutant proteins elute as monomers in the absence of nucleotide. In agreement with previous results, wild-type protein failed to form stable dimers in either 10 mM ATP or 10 mM ADP. In contrast, the E171Q MJ0796 mutant dimerized in an ATP-dependent fashion, eluting with a retention time corresponding to the predicted molecular weight of the MJ0796 dimer. The extent of dimerization increased as ATP concentration was varied from 0 to 10 mM (see Fig. 2 in Moody *et al.*, 2002). ADP inhibited this dimerization, and non-hydrolyzable ATP analogues only weakly promoted the shift from monomer to dimer. Similar gel filtration results were also obtained with the corresponding mutant form of MJ1267, E179Q (Moody *et al.*, 2002).

To obtain true equilibrium measurements of the NBD monomer–dimer transition, analytical ultracentrifugation experiments were performed. Wild-type MJ0796 failed to appreciably dimerize in the presence of ATP and EDTA, and the calculated K_D for momoner–dimer equilibrium is at least 150–500 μM protein at nucleotide concentrations up to 10 mM (unpublished results). This likely accounts for the fact that in the absence of the divalent cations necessary for hydrolysis, or even with non-hydrolyzable ATP analogues, wild-type NBD dimers have not been isolated. The hydrolysis deficient E171Q MJ0796, on the other hand, dimerizes in the ultracentrifugation experiments similarly as with gel filtration. The measured K_D for momoner–dimer equilibrium decreases from 200 μM in the absence of nucleotide to less than 100 nM at saturating ATP concentrations (Moody *et al.*, 2002). In both the ultracentrifugation and gel filtration experiments on E171Q MJ0796, the ATP concentration required for half maximal dimerization was calculated to be 50 μM (unpublished results), coinciding with the wild-type K_m for ATPase activity. Taken together, the experiments using the archaebacterial proteins MJ0796 and MJ1267 suggest that ATP-dependent dimerization of the nucleotide binding domains is a necessary step in the mechanistic cycle, and that the catalytically deficient mutants are trapped in an intermediate state immediately prior to ATP hydrolysis.

While the biochemical and structural characterization of MJ0796 and MJ1267 (Moody *et al.*, 2002) provided further evidence in support of the Rad50cd model of ABC transporter NBD dimerization, it was the crystallization of E171Q MJ0796 in dimeric form with two ATP molecules at the interface that settled the debate (Smith *et al.*, 2002). The details of the intersubunit interactions in the E171Q MJ0796 structure differed from those in Rad50cd, but the significant overall features were conserved, most notably the nucleotide binding pocket, composed of residues from both subunits, involving all three major conserved motifs; the Walker A and Walker B from one subunit, and the signature sequence LSGGQ from the opposing subunit. In addition to being the initial NBD from an ABC transporter crystallized in a dimer, the E171Q MJ0796 structure provided for the first time a comparison between both ATP- and ADP-bound states of the same NBD. The differences between the two MJ0796 structures match well with the earlier predictions based on the first two ADP-bound structures in comparison to ATP-bound HisP, namely the rotation of the α-helical subdomain (Hung *et al.*, 1998; Karpowich *et al.*, 2001; Yuan *et al.*, 2001). In addition, closely following Moody *et al.* arrived evidence that in *E. coli* MalK and human P-glycoprotein the LSGGQ motif is in close proximity to the nucleotide binding site based on photocleavage and chemical cross-linking experiments (Fetsch and Davidson, 2002; Loo *et al.*, 2002). In another significant step for the ABC transporter field, the *E. coli* half transporter BtuCD was crystallized shortly after the E171Q MJ0796 dimer, illustrating both the TMDs and NBDs in atomic detail (Locher *et al.*, 2002)

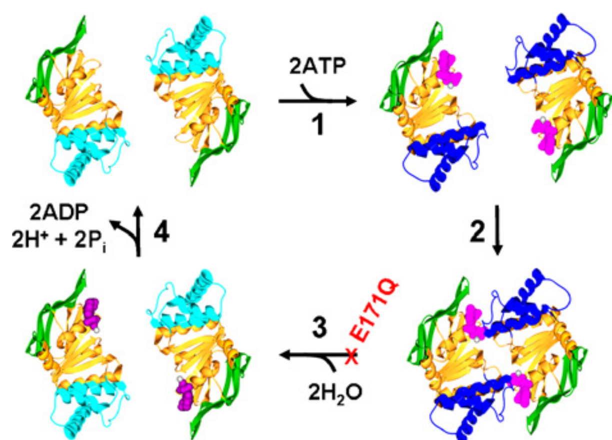

Fig. 1. A model for NBD interactions during the mechanochemical reaction cycle of ABC transporters. The F1-type core subdomain is shown in *yellow*, the anti-parallel β-sheet subdomain is shown in *green*, and the α-helical subdomain is shown in either *cyan* (nucleotide free and ADP-bound states) or *dark blue* (ATP-bound states). ATP is shown in *magenta*, and ADP is shown in *purple*. Step 1 combines ATP binding to NBD monomers with the α-helical subdomain rotation (signified by the change from *cyan* to *dark blue*). Step 2 rapidly follows as the NBDs dimerize. Step 3 combines ATP hydrolysis with the opposite α-helical subdomain rotation (signified by the reverse change from *dark blue* to *cyan*) to that exhibited during step 1 and subsequent dissociation of the NBD dimer into ADP-bound monomers. Step 4 illustrates the release of hydrolysis products and a return to the beginning of the reaction cycle. Any one or combination of the four steps may serve as the power stroke during the reaction cycle of ABC transporters. The catalytic carboxylate mutants (such as E171Q MJ0796) become arrested immediately following step 2 and cannot proceed through step 3. Crystal structures have been solved for the species in the lower half of the figure (the ADP-bound monomers and ATP-bound dimer).

and providing further evidence supporting the Rad50cd model.

Combining the biochemical and structural data available for ABC transporter NBDs, a mechanism was proposed (Fig. 1, adapted from Moody *et al*., 2002) in which nucleotide-free NBDs bind ATP, upon which the α-helical subdomains rotate in relation to the F1-type core subdomain. The ATP-bound subunits subsequently dimerize. This association (steps 1 and 2 in Fig. 1) may serve as the power stroke of ABC transporter function, harnessing the binding energy of ATP to do mechanical work, if one assumes the NBD dimerization transmits conformational changes through the TMDs. ATP hydrolysis then drives the NBD dimer apart, products are released, and the cycle begins anew with ATP binding to the nucleotide-free NBDs. In this scenario, ATP hydrolysis and/or product release (steps 3 and 4 in Fig. 1) could also serve as the power stroke for the transporter. It has been hypothesized that immediately following hydrolysis, the ADP remains bound to the Walker A motif of one monomer while the γ-phosphate remains bound to the LSGGQ motif of the opposing monomer, providing an electrostatic repulsion to drive apart the NBD dimer (Smith *et al*., 2002). It appears that binding at both sites is required for NBD dimerization (as we have illustrated in step 1 in Fig. 1), and for simplicity we have depicted two hydrolysis events in our mechanism, although a single hydrolysis event might be sufficient to disrupt the interface and return the NBDs to monomeric form. Efforts are ongoing in the field to determine if one or two hydrolysis events are necessary and sufficient for one complete transport cycle, as recent studies by Tampe and colleagues on TAP and by Senior and colleagues on Mdr3 have begun to address this question (Chen *et al*., 2003; Tombline *et al*., 2004a,b).

PROPOSED ABC TRANSPORTER MECHANISM AND IMPLICATIONS FOR CYSTIC FIBROSIS

This proposed mechanism fits well when one considers a homodimeric system in which two copies of the same NBD are utilized (i.e., MalK in maltose transport and HisP in the histidine permease), but ABC transporters such as those in the human ABCC subfamily present a conundrum. ABCC subfamily members such as the multidrug resistance-associated protein (MRP) (Cole and Deeley, 1998), CFTR (Lewis *et al*., 2004), and the sulfonylurea receptor (SUR) (Bryan and Aguilar-Bryan, 1999) possess two TMDs and two NBDs on the same polypeptide, with the N-terminal NBD1 possessing the canonical Walker A and LSGGQ motifs but missing the catalytic glutamate following the Walker B motif. The C-terminal NBD2 includes the Walker A and B motifs as well as the catalytic glutamate residue, but the LSGGQ motif is not strictly conserved. In the hypothetical ABCC NBD1–NBD2 heterodimer only one nucleotide binding site possesses a canonical arrangement with conserved residues from the three motifs, while the other binding site lacks the catalytic glutamate and the typical LSGGQ sequence. The question arises once again of how many hydrolysis events take place during each reaction cycle, as ABCC subfamily members appear to only contain one hydrolysis-capable site. A recent study of CFTR channel opening (Vergani *et al*., 2005) firmly establishes NBD dimerization as an integral aspect of ABC transporter function, and suggests that mutating the catalytic glutamate to glutamine (E1371Q, analogous to E171Q in MJ0796) in full-length CFTR results in a hyperstable NBD1–NBD2 heterodimer. While recent crystallization efforts have resulted in CFTR NBD1 structures (Lewis *et al*., 2004, 2005; Thibodeau *et al*., 2005), the field awaits a heterodimeric

NBD1–NBD2 structure and, ultimately, atomic detail of the full-length human transporter that promise to shed further light on ABC transporter functional mechanism and provide novel approaches for the treatment for cystic fibrosis.

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