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Genomics and the mechanism of P-glycoprotein (ABCB1)

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Abstract The development of effective clinical interventions against multidrug resistance (MDR) in cancer remains a significant challenge. Single nucleotide polymorphisms (SNPs) contribute to wide variations in how individuals respond to medications and there are several SNPs in human P-glycoprotein (P-gp) that may influence the interactions of drug-substrates with the transporter. Interestingly, even some of the synonymous SNPs have functional consequences for P-gp. It is also becoming increasingly evident that an understanding of the transport pathway of P-gp may be necessary to design effective modulators. In this review we discuss: (1) The potential importance of SNPs (both synonymous and non-synonymous) in MDR and (2) How new concepts that have emerged from structural studies with isolated nucleotide binding domains of bacterial ABC transporters have prompted biochemical studies on P-gp, leading to a better understanding of the mechanism of P-gp mediated transport. Our results suggest that the power-stroke is provided only after formation of the pre-hydrolysis transition-like (E·S) state during ATP hydrolysis.

Keywords ATP-binding cassette · P-glycoprotein · Multidrug resistance · ATP hydrolysis · Catalytic/transport pathway · Single nucleotide polymorphism

Abbreviations

ABC	ATP-binding cassette
[^{125}I]IAAP	[^{125}I] Iodoarylazidoprazosin
NBD	nucleotide binding domain
P-gp	P-glycoprotein
TMD	Transmembrane domain
Vi	orthovanadate

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Multidrug resistance and P-glycoprotein

Last year marked the 30th anniversary of the discovery that P-glycoprotein (P-gp) is expressed in multidrug resistant cell lines (Juliano and Ling 1976), and also the 20th anniversary of the cloning and sequencing of the human *MDR*1 gene (Chen et al. 1986). It is now almost universally accepted that membrane transporters such as P-gp play a significant role in the development of multidrug resistance (MDR), wherein cancer cells develop simultaneous resistance to many chemically unrelated compounds and to natural product anti-cancer agents (Gottesman and Ling 2006). The clinical ramifications of MDR can be quite fatal. Six essential hallmarks of cancer have been described (Hanahan and Weinberg 2000): (1) self-sufficiency in growth signals, (2) insensitivity to growth-inhibitory (anti-growth) signals, (3) evasion of programmed cell death (apoptosis), (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis. From the perspective of a clinician, tumors that show the first five characteristics are dangerous but treatable. On the other hand, nine out of ten deaths that occur due to cancer result from tumors that have metastasized. To a large extent this is because for metastatic tumors chemotherapy is often the only treatment option and MDR presents a major obstacle (Gottesman et al. 2002). Pharmacological modulation of MDR has been attempted since 1981 (Tsuruo et al. 1981) using agents such as verapamil that inhibit the drug

transporters. However, despite several generations of MDR modulators that have gone through clinical trials, none has made it into the clinic. It has been suggested recently that it may be time to design inhibitors of P-gp based on what has been learned about the mechanism of this transporter (McDevitt and Callaghan 2007).

P-gp is a typical ATP-binding cassette (ABC) transporter protein composed of two homologous halves, each containing a transmembrane domain (TMD) and a nucleotide-binding domain (NBD), separated by a flexible linker region. Interaction of the two halves of P-gp is critical for the functioning of the molecule, and the flexible linker region is necessary for the proper interaction of the two halves, most likely for the communication between the two ATP sites (see Ambudkar et al. 2003 and references therein). There is also considerable evidence that suggests that the two ATP sites of P-gp show alternate catalysis (Senior et al. 1995). Mutational analyses, labeling with the photoaffinity substrate analogues, and crosslinking with the thiol reactive substrate derivatives suggest that the drug-binding domains reside in the transmembrane helices contributed by both halves (transmembrane helices 4–6 and 10–12) (Loo and Clarke 2005). Early models predicted that the energy of ATP hydrolysis was coupled to the transport of drug-substrate. In recent years, numerous x-ray crystal structures of isolated NBDs of ABC proteins have been solved. This has led to the emerging concept of an ATP-driven dimerization of the two NBDs to form an ATP sandwich (reviewed in Sauna and Ambudkar 2007), and has prompted further biochemical studies and a reexamination the transport pathway of P-gp (see below).

It is also important that careful consideration be given to single nucleotide polymorphisms (SNPs) in P-gp. Genetic variability is high in human P-gp and specific polymorphisms and haplotypes occur at high frequencies in certain populations (Ambudkar et al. 2003). In addition, a recent report indicates that not only non-synonymous but also synonymous SNPs in P-gp can influence substrate specificity (Kimchi-Sarfaty et al. 2007). Thus these considerations should be part of any drug development effort.

SNPs in human *MDR*1: synonymous SNPs have functional consequences

The human *MDR*1 or *ABCB*1 gene is over 120 kb and contains 28 exons and the coding region accounts for less than 5% of the total (Sakaeda 2005). The gene lies on chromosome 7 at q21.1 (Callen et al. 1987). Polymorphisms have been reported in the *MDR*1 gene since 1989 (Kioka et al. 1989), however the first systematic screening was performed by Hoffmeyer et al. in 2000 and 15 SNPs were detected (Hoffmeyer et al. 2000). Subsequently over 50 SNPs have been identified in the human *MDR*1 gene (Ambudkar et al. 2003). A surprising finding by Hoffmeyer et al. was that a polymorphism, C3435T in exon 26, which does not result in an amino acid change, was associated with duodenal expression of *MDR*1 and thereby intestinal absorption of digoxin, a substrate of P-gp. This polymorphism has been extensively studied and the interethnic differences in the frequency of this polymorphism and the effects of the mutation on expression have been summarized by Sakaeda (2005). Another frequent SNP 1236C>T is also synonymous and interestingly both 1236C>T and 3435C>T are part of the most common haplotype observed in the human MDR1 gene, 1236C>T/2677G>T/3435C>T, which in ethnic groups such as Chinese, Malay and Indian represents 31–49% of the population (Pauli-Magnus and Kroetz 2004). The fact that this haplotype represents a large fraction of certain populations raises two important questions: (1) What selective advantages does this haplotype confer? (2) Does the haplotype (or the individual SNPs) affect the MDR phenotype? To determine whether this haplotype affects the MDR phenotype, the three SNPs individually and as a haplotype were characterized in a transient expression system (Kimchi-Sarfaty et al. 2007). All variants were expressed at equivalent levels and showed comparable transport function when measured with several fluorescent substrates using flow cytometry. There was, however, a reduction in the extent of reversal of transport by the MDR modulators verapamil and cyclosporine A in the haplotype. Current dogma would have had us predict that this change in phenotype would be caused by the non-synonymous SNP. The surprising observation, however, was that the non-synonymous SNP alone had no effect; the next obvious question was how the synonymous SNPs 1236C>T and 3435C>T alter the interaction of P-gp with some modulators (albeit in combination with a non-synonymous SNP). Splice variants and mRNA stability could explain this observation, but a full-length mRNA was found and its levels and P-gp protein levels and localization were unchanged in the haplotype. Alternative amino acids could, in theory, be introduced as substitutes for the amino acids encoded by the haplotype protein. However, protein sequencing of the wild-type P-gp and the haplotype by mass spectrometry resulted in identification of 82 peptides showing that each of these sequences was identical in both cases. These observations suggested that splice-variant alterations in mRNA stability and encoded amino acid substitutions were unlikely explanations for the P-gp phenotype. The use of the conformation-sensitive antibody against P-gp, UIC2, and limited trypsin digestion showed that there were differences between the wild-type and the haplotype P-gp's, suggesting that it was plausible that there were subtle but measurable differences in the conformation at sites where drugs and modulators interact. An intriguing

aspect of the haplotype is that the three SNPs represent codons that are rarer than those of the wild-type P-gp. It has therefore been hypothesized that the introduction of rare codons results in a translation pause and an alteration in the rate of translation affects the final tertiary structure of the protein (see Sauna et al. 2007 for a detailed discussion of this hypothesis). Although this study did not directly demonstrate that codon usage affects the rate of translation, when codons more rare than those found in the original haplotype were introduced, the result was even more marked changes in inhibitor sensitivity. These studies show that it is imperative to pay more attention to synonymous SNPs in the development of pharmacological interventions against MDR than for many other protein targets. Subtle changes in the tertiary structure would be construed to represent a misfolded protein and eliminated by the quality control machinery of the cell in the case of most proteins. P-gp (and other multidrug transporters), on the other hand, transports a broad range of amphipathic compounds (Gottesman et al. 2002; Ambudkar et al. 2006a, b) and it is likely that some structural flexibility at the transport substrate site may be favored, and therefore not eliminated. P-gp molecules with alternative tertiary structures and different drug-substrate (and/or modulator) specificity arising from SNPs or haplotypes would result in variable responses in the clinic.

The nucleotide binding domains of P-gp and the conserved sub-domain A-loop

P-gp has the predicted 2D structure of a typical ABC protein, two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs). The drug-binding sites are located on the TMDs and show little sequence homology, whereas the NBDs exhibit very high sequence homology. The crystallization and structure determination of isolated NBDs from bacterial and eukaryotic sources have provided important information on the organization of the ATP-binding site(s). The biochemical roles of several of the conserved amino acids in the NBDs have been elucidated using site-directed mutants of P-gp (see Table 1 in Ambudkar et al. 2006a).

The Walker A and Walker B motifs of P-gp are involved in the nucleotide binding. A conserved D residue in the Walker B motif (at positions 555 and 1,200 of human P-gp) plays a role in the coordination of Mg^{2+} (Hrycyna et al. 1999). An adjacent glutamate residue is often referred to as the catalytic carboxylate base. Though the precise role of this residue is still in dispute (Zaitseva et al. 2005a, b), it is critical for the ATP hydrolysis (Sauna et al. 2002, 2006). The H-loop or switch region was also shown to act as a catalytic base bringing about ATP hydrolysis via substrate-assisted catalysis (Zaitseva et al. 2005a, b). The signature region, a hallmark of ABC transporters, appears to be critical for ATP hydrolysis as well as communication with TMDs (Hrycyna et al. 1998). The other diagnostic marker of ABC transporters, the D-loop, was also suggested to be involved in communication between the catalytic sites (Jones and George 2004). The structure of the NBD (HisP) of the histidine permease of *Salmonella typhimurium* (Hung et al. 1998) suggested that the conserved Q residue (that characterizes the Q-loop) may play a role in the hydrolysis of the γ-phosphate of ATP. However, site directed mutagenesis studies in mouse P-gp showed that this residue has no role in hydrolysis *per se*, but mutations of this residue impaired the communication between TMDs and NBDs (Urbatsch et al. 2000a, b).

Recently, Mao et al. (2004) reported that aromatic amino acid residues interact with the adenine ring of ATP via π-π stacking, cation-π interaction, or hydrogen bonding according to analysis of high-resolution structures of nucleotide-binding proteins. We examined whether these aromatic residues represent a conserved sub-domain (Kim et al. 2006). This aromatic residue, 25±2 amino acids upstream of the Walker A motif, is conserved in 16,312 (88.1%) of ABC proteins by sequence alignment of 18,514 ABC domains with the consensus conserved sequence in a database of all non-redundant proteins. In P-gp, these conserved aromatic residues correspond to Y401 and Y1044. We generated mutants where the Y residue was substituted with F, W, C, L, or A. None of these mutations affected the expression of protein. Although the mutants Y401F, Y401W, Y1044F and Y1044W transported fluorescent substrates similar to wild-type protein, there was a significant decrease (2–2.5 fold) in the affinity of the nucleotide for the Y401W mutant P-gp. Y401C and Y401L mutant P-gps showed partial (30–50%) transport function. However, transport was completely abolished in Y401A, Y1044A, and Y401/Y1044A mutant P-gps and there was no detectable binding of 8-azido[α-^{32}P]ATP or TNP-ATP, Vi-induced trapping of nucleotide, and ATP hydrolysis in these mutant P-gps. Homology modeling of the NBDs of P-gp based on the structure of a dimer of the E171Q mutant MJ0796 NBD supports the view that Y401 stacks with the adenine ring of ATP, most likely through π–π interactions. In addition, several crystal structures of NBDs of ABC transporters showed interaction between the adenine ring of the nucleotide and aromatic residues (see for example Hung et al. 1998; Locher et al. 2002; Smith et al. 2002; Chen et al. 2003; Dawson and Locher 2006; Zaitseva et al. 2006). Therefore, this sub-domain should be considered as an integral part of the NBD in ABC domains and we have named this sub-domain the A-loop (*A*romatic residue interacting with the *A*denine ring of *A*TP; see Ambudkar et al. 2006b; Kim et al. 2006 and Fig. 1a).

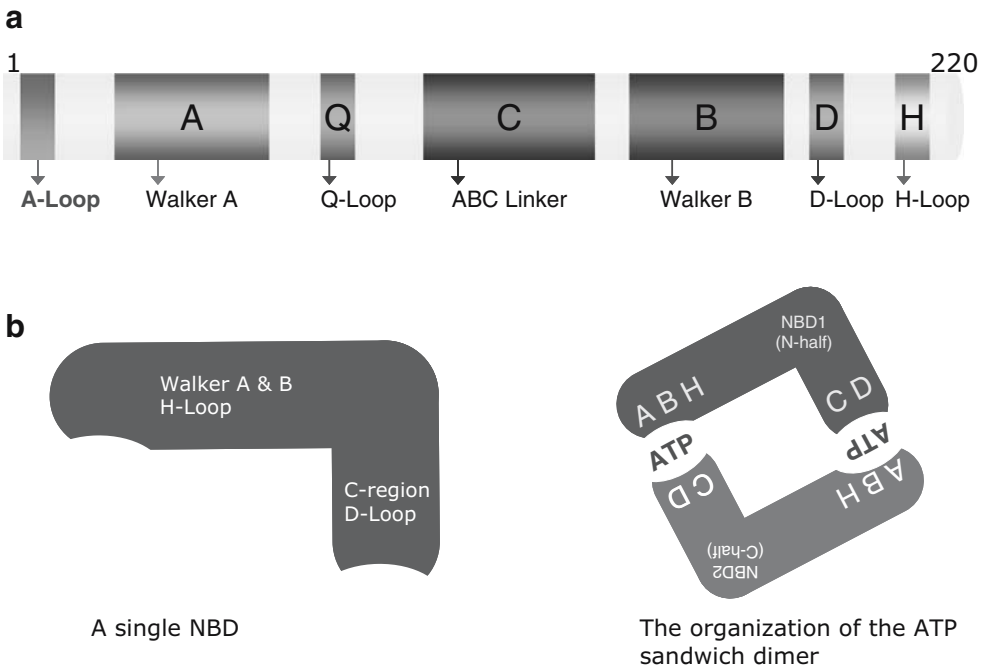

Fig. 1 Organization of the ATP-binding site. (**a**)Schematic showing the conserved domains with each NBD of ABC transporters, including the recently identified sub-domain, the A-loop (Kim et al. 2006). (**b**) The two NBDs interact to form a functional ATP site. The ATP is sandwiched between the Walker A and B domains and the H-loop of one NBD and the C-region and D-loop of the other NBD. Both NBDs of P-gp have canonical consensus sub-domains

The occluded nucleotide conformation of P-gp and the transport mechanism

A crystal structure at 1.5 Å resolution of the HisP (the ATP-binding subunit of the histidine permease) was the first high resolution structure of the NBD of an ABC transporter (Hung et al. 1998). This structure depicts a monomer, and the ATP binding site shows limited hydrogen bonding and electrostatic interactions with ATP. Well before the resolution of this structure, there was abundant biochemical evidence that a functional ABC transporter is composed of two NBDs and two TMDs (for review see Ambudkar et al. 1999). Based on the crystal structure of HisP and other ATP binding proteins as well as sequence alignments of ABC proteins, Jones and George postulated that in an NBD dimer, the ATP is sandwiched between the Walker A, Walker B, Q- and H-loops of one NBD and the D-loop and signature sequence of the apposing NBD (see Jones and George 1999 and Fig. 1b). Subsequent to this prediction, the structure of a stable NBD dimer of Rad50, formed in the presence of the nonhydrolyzable ATP analog AMPPNP, confirmed the existence of such an architecture (Hopfner et al. 2000). The crystal structure of the Rad50 dimer shows two AMPPNP molecules sandwiched between the Walker A motif of one subunit and the LSGGQ signature motif of the other NBD. It must however be noted that Rad50, which is a DNA repair enzyme, is only remotely related to ABC transport proteins and the structure of its NBDs has diverged considerably from that of true ABC transporters. Most notably, although Rad50 carries the LSGGQ sequence, its position is different from that in an ABC transport protein (Smith et al. 2002). Subsequently, Thomas and coworkers observed that an E→Q mutation of a highly conserved glutamate in the Walker B region of ABC proteins results in ATPase activity being severely impaired and isolated a mutant NBD that formed stable dimers in the presence of ATP (Moody et al. 2002). Using this strategy, Hunt and coworkers solved the crystal structure of the E171Q mutant of MJ0796 as a dimer with two ATP molecules (Smith et al. 2002). This structure exhibits the most important features observed in the Rad50 dimer and it is important to note that the Walker A and B and the opposing Signature sequence residues do not interact directly with each other but via the ATP molecule that is sandwiched between the two faces of the cavity. Crystal structures of isolated NBDs of several ABC transporters (for a recent review see Sarkadi et al. 2006) suggest that the interaction of the two NBDs in a head-to-tail configuration may be a common feature of this family of membrane proteins (Fig. 1b). In recent years, several groups have also obtained structures of isolated NBDs that elucidate the interdomain movements that accompany ATP binding and hydrolysis. Thus for example structures of the MalK (the NBD subunit of the *E. coli* maltose transporter) have been obtained in the open, semi-open and closed conformations (Chen et al. 2003) as well as in the ATP-bound and ADP-bound states (Lu et al. 2005). Studies such as these show that ATP-driven dimerization is accompanied by rigid body rotations, primarily of the helical subdomains within the NBDs. Although isolated NBDs have provided important structural and biochemical information, such a system cannot address two important questions: (1) How is

the catalytic cycle of ATP hydrolysis coupled to conformational changes at the TMDs (where the transport occurs)? (2) Do the TMDs exert a regulatory effect on the NBDs?

Several laboratories have studied in detail substitutions at the highly conserved glutamate reside in the Walker B region of mouse and human P-gps. These glutamate residues are at positions 556 and 1201 in human P-gp and 552 and 1197 in mouse P-gp. Moreover, these glutamates are equivalent to the E171 residue of MJ0796 which was substituted to provide the first biochemical and structural evidence for an ATP sandwich dimer in an ABC protein (Moody et al. 2002; Smith et al. 2002). Individual substitutions of the conserved glutamate in the two NBDs of mouse P-gp (E552Q and E1197Q) resulted in a drastic reduction in basal ATPase activity and no stimulation in the presence of drug-substrates (Urbatsch et al. 2000a, b), similar results were obtained with human P-gp (Sauna et al. 2002). In addition, we characterized the double mutant (E556Q/E1201Q) where the glutamates in both NBDs were simultaneously mutated (Sauna et al. 2002). When this mutant was incubated at 37 °C with 8-azido-[α-^{32}P]ATP, we found that the nucleotide was occluded in a non-exchangeable form in the absence of any transition state analog like orthovanadate (Vi) or beryllium fluoride (BeFx). Additional studies from our laboratory (using the E556Q/E1201Q mutant of human P-gp) demonstrated that the occlusion of ATP is strongly temperature-dependent and that the occluded nucleotide conformation shows reduced binding of the transport-substrate [^{125}I]iodoarylazidoprazosin ([^{125}I]IAAP) (Sauna et al. 2006). It is important to understand what these results mean in the context of the catalytic cycle of P-gp-mediated ATP hydrolysis.

Figure 2 depicts a scheme to describe the catalytic cycle of P-gp-mediated ATP-hydrolysis. The P_i analog, Vi, has long been used to trap the post-hydrolysis reaction intermediate, P-gp·MgADP·Vi (for review see Sauna et al. 2001) which represents the enzyme–product (E·P) state of the reaction. Numerous studies have shown that as the release of P_i is necessary for Vi to trap P-gp in this ternary complex, it is always MgADP (or other nucleoside diphosphate) that is found in the Vi-trapped state of P-gp (for review see Sauna et al. 2001). Double mutants of the conserved glutamate, however, are hydrolysis deficient and cannot generate ADP and occlude ATP in a pre-hydrolysis intermediate, P-gp(E556Q/E1201Q)·MgATP (see Fig. 2) and represent the enzyme–substrate (E·S) state. Senior coined the term, "occluded nucleotide conformation" (Tombline and Senior 2005) to distinguish this Vi or BeFx-independent "occlusion" of ATP in the mutant of P-gp from the "Vi-trapped" state in wild-type P-gp. Both the Senior group and we have demonstrated that unlike the ATP-driven dimers obtained with isolated NBDs, which exhibit a stoichiometry of 2 mol ATP per dimer (see for example, Smith et al. 2002), P-gp shows a maximal

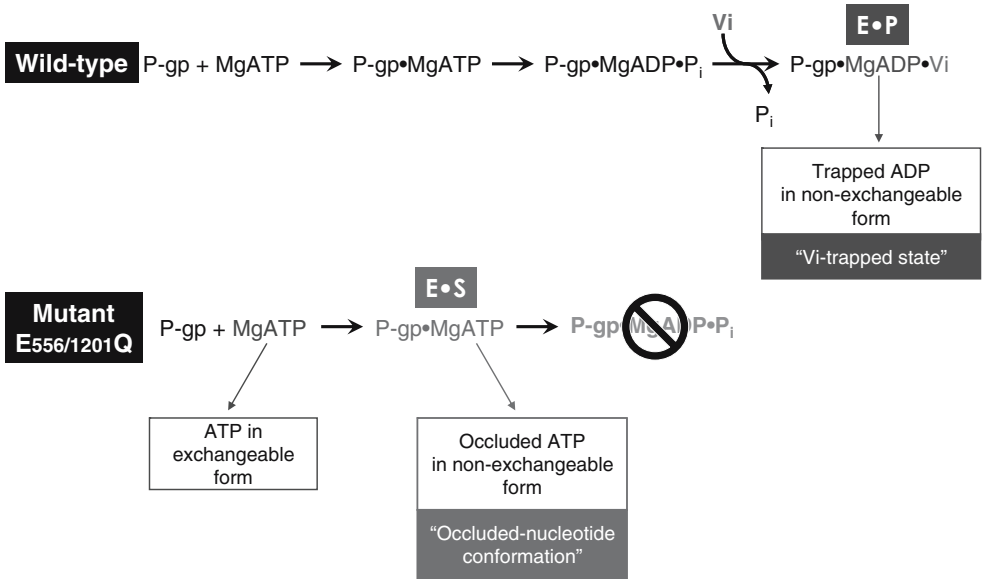

Fig. 2 Schematic showing the ATPase reaction in wild-type and mutant (E556Q/E1201Q) P-gps. The upper panel shows that in wild-type P-gp, following ATP hydrolysis,Vi, an analog of P_i, "traps" MgADP in a non-exchangeable form in the P-gp·MgADP·Vi ternary complex. The E556Q/E1201Q mutant P-gp shows minimal ATP hydrolysis and "occludes" MgATP in a non-exchangeable form. Note that when ATP initially binds to P-gp it is freely exchangeable. This is followed by an energy driven conformational change that occludes the MgATP. The Vi-induced trapping of MgADP in wild-type represents a post-hydrolysis E·P state, whereas the mutant P-gp traps MgATP in a pre-hydrolysis E·S state. The occlusion of nucleotide in the ES reaction intermediate is also highly temperature-dependent with an E_{act} of ~ 60 kJ/mol. The occluded or Vi-trapped nucleotide cannot be exchanged in the presence of 30,000-fold excess ATP (Sauna et al. 2006)

stoichiometry of 1 mol ATP occluded per mol P-gp (Tombline et al. 2004; Sauna et al. 2006). This may represent a distinction between P-gp and the other transporters studied or a universal feature of full-length ABC proteins in the presence of the membrane-spanning TMDs. Our observation that ATP occlusion (in mutant P-gp) is strongly temperature-dependent, unlike ATP binding in wild-type P-gp (Sauna et al. 2006), suggests that formation of the occluded nucleotide conformation involves conformational changes that follow ATP binding. Thus it is plausible that nucleotide binding initially leads to a symmetric dimer with ATPs bound at both NBDs which progresses to an asymmetric occluded state where one of the two ATPs is tightly bound in a non-exchangeable form (see Fig. 1 in Sauna and Ambudkar 2007). Structural and biochemical evidence from studies with isolated NBDs had prompted the idea that NBDs join together upon binding ATP and then dissociate upon hydrolysis (Janas et al. 2003; Lu et al. 2005). However, the recently solved structure of a full-length ABC protein Sav1866, a multidrug transporter from *S. aureus* (Dawson and Locher 2006), suggests that such a mechanistic scheme is unlikely (Schuldiner 2006). The novel feature of this structure is that transmembrane helices are not aligned side by side but are intricately interleaved and their maximal separation during the catalytic cycle is likely to be limited. Moreover, the close proximity of the two NBDs suggests that the differences between the "open" and "closed" dimers may be more subtle than complete dimer association and dissociation (Higgins 2007). The hypothesis that the symmetric binding of two ATPs is followed by the asymmetric occlusion of one of the two ATPs is also favored by a molecular dynamics simulation of the ATP binding process in BtuCD (an ABC protein which is the vitamin B_{12} importer in *E. coli*) (Oloo and Tieleman 2004). Our studies with the P-gp mutant (E556Q/E1201Q) also allow us to address the question of what role, if any, the occluded nucleotide conformation plays in the transport pathway. We have demonstrated that occlusion of [α-^{32}P]ATP and that the formation of the occluded dimer is accompanied by a temperature-dependent reduced binding of the drug-substrate analog [^{125}I]IAAP (Fig. 3 and Sauna et al. 2006). The former suggests that occlusion of ATP is accompanied by conformational changes at the NBDs and the latter that these conformational changes are transmitted to the TMDs. These studies are consistent with earlier work that demonstrated that incubation of wild-type P-gp at 37°C with non-hydrolyzable analogs of ATP resulted in a conformation with reduced affinity for P-gp drug-substrates such as [^{3}H]-vinblastine (Martin et al. 2000; Rosenberg et al. 2001). The formation of the occluded nucleotide conformation of the mutant human P-gp, E556Q/E1201Q, has an activation energy of ~60 kJ/mol and the activation energies for nucleotide trapping and decrease in drug binding are equivalent (Sauna et al. 2006). This suggests that the occlusion of ATP and the high affinity→low affinity switch at the drug-binding site are also coupled (see Fig. 3). It must however be noted that binding of nucleotide in the absence of occlusion does not result in a high affinity to low affinity switch at the drug-binding site (Sauna and Ambudkar 2000, 2001).

Conclusions

A substantive body of work now exists that suggests that SNPs in the human *MDR*1 gene (both synonymous and non-synonymous) can affect protein levels as well as

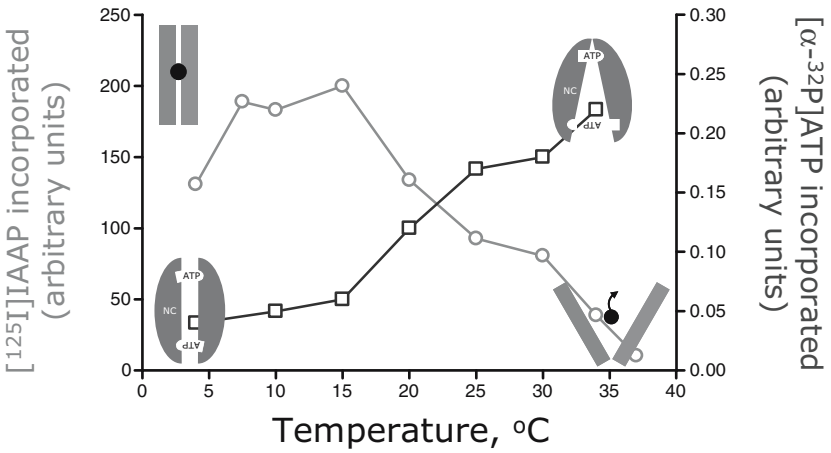

Fig. 3 Occlusion of ATP in the mutant P-gp (E556Q/E1201Q) is accompanied by a high-affinity to low affinity switch at the TMDs. The E556Q/E1201Q mutant P-gp occludes [α-^{32}P]ATP in a temperature-dependent manner (*black squares*). In the same temperature range there is a concomitant decrease in the binding of the transport-substrate [^{125}I]IAAP when the mutant P-gp is incubated with 1 mM ATP (*gray circles*). The superimposed illustrations depict occlusion of ATP (*in black*) at the NBDs and the high-affinity to low affinity switch at the drug-substrate site in the TMDs (*in gray*). See Sauna et al. (2006) for experimental details

conformation. Future studies should systematically define the effect of the more common P-gp SNPs and haplotypes on the pharmacokinetics of MDR modulators and a repertoire of anticancer drugs in in vitro studies. These approaches would help to design clinical studies with more stringent inclusion-exclusion criteria that include genetic profiling. Such studies are more likely to meet with success than the current more broad-based approaches. Concomitantly, the last few years have seen significant advances in our understanding of the catalytic cycle of ABC transporters. The concept of an ATP-driven dimerization of the two NBDs and consequences of this early event on the transport pathway has had a large impact on our understanding of the catalytic cycle of ABC proteins. P-gp has proved to be a good model system to extend our understanding of how formation of the ATP sandwich may be coupled to conformational changes at the drug-substrate sites in the TMDs. Our recent work demonstrates that the power-stroke for the conformational change in the transmembrane region resulting in a decrease in the affinity of transport-substrate for P-gp is provided at the pre-hydrolysis (E·S) reaction intermediate and not just by the binding of the nucleotide alone (Sauna et al. 2006; Sauna and Ambudkar 2007).

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