Chapter 4
Structures and Transport Mechanisms of the ABC Efflux Pumps

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Abstract The ATP-binding cassette (ABC) transporters form one of the largest families of proteins in living organisms. They are overrepresented in bacteria where they are involved in the influx or efflux of various molecules. Although bacterial drug efflux transporters were initially discovered as ion-motive-driven pumps, evidence has accumulated since the mid-1990s that members of the ABC superfamily can play a prominent role in drug resistance mechanisms. Yet, the implication of drug efflux ABC transporters in clinical settings is still lagging behind for most bacterial pathogens. Thanks to the accumulation of three-dimensional structures, our knowledge of the functioning mechanisms of drug efflux transporters has progressed tremendously in the recent years, but many questions still remain. In this chapter, we will summarize the current view of the structures and transport mechanisms of drug efflux ABC transporters with an emphasis on multidrug bacterial efflux pumps. Unsolved mysteries about these fascinating transporters will also be mentioned.

Keywords ABC transporters • Multidrug resistance • P-Glycoprotein • ATP switch model • Constant contact model • Drug binding • BmrA • LmrA • MacAB • MsbA • PatAB • Sav1866

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X.-Z. Li et al. (eds.), *Efflux-Mediated Antimicrobial Resistance in Bacteria*,
DOI 10.1007/978-3-319-39658-3_4

4.1 Introduction

The ATP-binding cassette (ABC) transporters form a large superfamily of proteins that use the energy of ATP binding and hydrolysis to translocate a wide variety of solutes across biological membranes [1]. (Although this chapter is focused on bacterial transporters, some references to eukaryotic transporters will also be included when relevant to this topic.) Apart from a few exceptions [2], the importers are only present in prokaryotes, while exporters are found in all organisms. The minimal functional unit contains two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) [3, 4]. These domains can be synthesized as four separate polypeptides or as various combinations of three, two, or a single polypeptide. The NBDs energize the transporter by binding and hydrolyzing ATP, while the TMDs are responsible for the specificity and translocation pathway of the substrates. Therefore the NBD primary sequences are fairly well conserved, while the TMD sequences and topology, in particular the number of transmembrane helices, are highly variable depending on the solute transported. In addition, the majority of the importers require an extracellular substrate-binding protein that delivers the substrate to the transporter [5].

4.2 Multidrug Transporters

4.2.1 Homodimers

The first bacterial multidrug resistance (MDR) ABC transporter was discovered in *Lactococcus lactis* and was named LmrA (*Lactococcus* multidrug resistance ATP) [6]. LmrA functions as a homodimer [7], each monomer being made of one TMD containing six predicted transmembrane helices and one ABC domain. LmrA was initially chosen for investigation based on its homology with the human MDR transporter P-glycoprotein. It was later shown to complement the P-glycoprotein human gene in eukaryotic cells [8]. The overexpression of LmrA in a drug-hypersusceptible strain of *Escherichia coli* induced a resistance phenotype to several structurally unrelated compounds, i.e., ethidium, daunorubicin, rhodamine 6G, and tetraphenylphosphonium [6]. In addition, the accumulation of daunorubicin in inverted membrane vesicles was dependent on ATP hydrolysis and inhibited by the other substrates and reserpine, a classical inhibitor of drug efflux pumps.

A *Bacillus subtilis* transporter homologous to LmrA was shown to transport a variety of drugs (e.g., Hoechst 33342, doxorubicin, and 7-aminoactinomycin D) when overexpressed in inverted *E. coli* membrane vesicles. Originally known as YvcC [9], this homodimeric transporter [10] was renamed BmrA (*Bacillus* multidrug resistance ATP) [11]. Later, a *B. subtilis* strain resistant to the antibiotic cervimycin C was isolated and shown to strongly upregulate the expression of *bmrA* due to a promoter mutation [12].

Two other drug transporters, Sav1866 from *Staphylococcus aureus* and MsbA from various bacteria, will be described in more details below.

4.2.2 Heterodimers

While LmrA and BmrA are typical homodimers, other drug transporters are heterodimers. The latter transporters include LmrCD in *L. lactis* [13], BmrCD in *B. subtilis* [14], PatAB in *Streptococcus pneumoniae* [15], and SmdAB in *Serratia marcescens* [16]. In these heterodimers, one of the ATP-binding sites is degenerated [17, 18] with conserved residues such as the glutamate adjacent to the Walker B motif, the histidine of the H-loop, and/or some residues in the signature motif of the opposite NBD being naturally substituted by non-consensual residues. Consequently, the functioning mechanism of these heterodimers is asymmetric with the degenerated NDB being poorly active in ATP hydrolysis. A similar scenario occurs in eukaryotic transporters that bear two nonequivalent NBDs (e.g., multidrug resistance protein MRP1, cystic fibrosis transmembrane conductance regulator CFTR, or antigen peptide transporter TAP1/TAP2) [19, 20].

LmrCD was shown to be a major MDR transporter in *L. lactis* [21, 22] and its expression is under the control of the transcriptional repressor LmrR [23, 24]. Binding of drugs to LmrR reduces its affinity for LmrCD promoter thereby inducing the expression of the MDR transporter [25–27].

BmrCD is a *B. subtilis* transporter whose expression is stimulated by various antimicrobial agents [14], especially protein synthesis inhibitors. The latter drugs were recently shown to induce the expression of BmrCD through a ribosome-mediated transcriptional attenuation mechanism [28]. When overexpressed in *E. coli* membranes, BmrCD transports several drugs such as Hoechst 33342, doxorubicin, and mitoxantrone [14]. Its efficient expression and purification was exploited for several structural and functional studies [29–31].

The implication of the *S. pneumoniae* transporter PatAB in MDR was first demonstrated when inactivation of its genes induced an increased susceptibility to several antimicrobial agents: acriflavine, berberine, ethidium bromide, and norfloxacin [32]. After exposing a laboratory strain to ciprofloxacin, a multidrug-resistant strain was isolated in which PatA and PatB genes were upregulated [33]. Such upregulation by fluoroquinolones was also found in clinical isolates [34–36]. Importantly, disruption of PatA and PatB genes overexpressed in many clinical isolates restored drug susceptibility, either completely for ethidium bromide or partially for fluoroquinolones [35]. Several mechanisms were described for PatAB upregulation: disruption of a transcriptional attenuator [37], gene duplication [38], or promoter region and internal mutations [39]. Studies of the transporter overexpressed in *E. coli* show that only the heterodimer is functional for drug efflux [40].

Other transporters whose function is less characterized were shown to have drug transport capabilities, such as TmrAB in *Thermus thermophilus* [18] or TM287/

TM288 in *Thermotoga maritima* [41]. A recent study suggests that TmrAB may be a glycolipid flippase analogous to the transporter MsbA [42].

4.2.3 Other Drug ABC Exporters with a Different Topology

DrrAB from *Streptomyces peucetius* exports the anticancer antibiotics daunorubicin and doxorubicin that this microbe produces. It was long thought to be a narrow-spectrum drug transporter until a recent biochemical characterization indicated its ability to also transport the Hoechst 33342 and ethidium bromide [43]. In contrast to LmrA and BmrA, where each monomer is a TMD fused to an NBD, DrrA is a single NBD subunit, while DrrB is a TMD subunit predicted to contain eight transmembrane helices [44].

MacAB-TolC from *E. coli* was characterized as a macrolide-specific tripartite efflux pump [45]. Homologues are present in various Gram-negative bacteria. MacB is an ABC transporter from the cytoplasmic membrane with an inverted topology: an *N*-terminal NBD is fused to a *C*-terminal TMD containing four predicted transmembrane helices. TolC is an outer membrane channel protein, while MacA is a membrane fusion protein that interacts with both partner proteins. Nanomolar affinity interactions occur between TolC and MacA and between MacA and MacB [46]. MacB is a dimer [47] whose ATPase activity is strongly stimulated by MacA [48]. Several drug-unrelated physiological functions have been proposed, the latest being protoporphyrin efflux [49].

4.3 Structure of the Nucleotide-Binding Domains, Consensus Motifs, and the ATP Sandwich Dimer

The NBDs of ABC transporters are well conserved, both in sequence with several motifs and in structure [50]. HisP, which is the ATPase subunit from a bacterial histidine importer, was the first NBD crystallized [51]. The domain had an L shape and is made of three subdomains (Fig. 4.1) [52]. One is a RecA-like subdomain present in many ATPases and that carries the Walker A and Walker B motifs [53, 54] as originally described in numerous ATPases (e.g., the Fo-F1) [55]. The former, also known as the P-loop, has the consensus sequence GX_2GXGKT/S (where X is any residue; see Fig. 4.1). Some backbone amino groups within this motif, and especially the ε-amino group of the conserved lysine, stabilize the bound nucleotide by making hydrogen bonds with the β- and γ-phosphate oxygen atoms. The Walker B motif is usually made of four hydrophobic residues that form a β-strand and is terminated by a conserved Asp. This Asp residue coordinates the catalytic Mg^{2+} cofactor by hydrogen bonding via a water molecule.

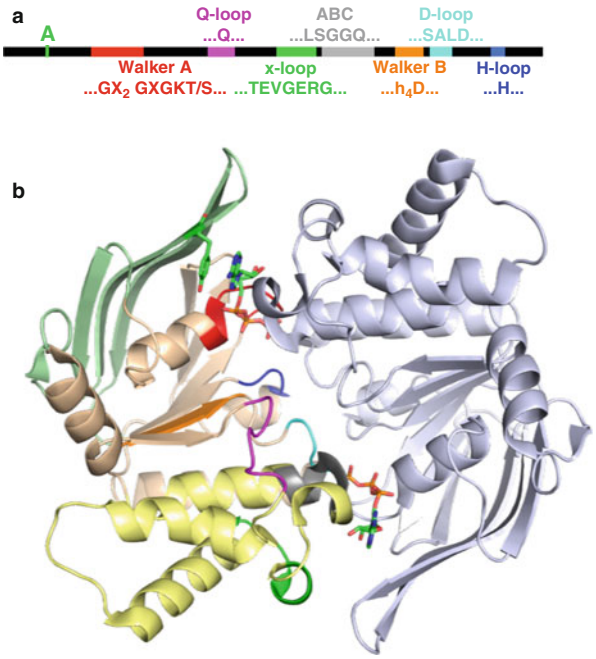

Fig. 4.1 Structure of the nucleotide-binding domains. (**a**) Schematic view of the conserved elements present in the NBD of exporters (see the text for details). The color-coding is the same as in (**b**). (**b**) The dimer of NBDs trapped in a transient ATP-bound state. The two identical NBDs of LolD (PDB code MJ0796) are shown here, and one is colored in gray and the other is colored in pale green, wheat, and pale yellow for the β-, RecA-, and α-helical subdomains, respectively. The conserved motifs are shown for one monomer in *red* (Walker A), *orange* (Walker B), *blue* (H-loop), *magenta* (Q-loop), *gray* (ABC signature), *green* (x-loop), and *cyan* (D-loop). The tyrosine which is part of the A-loop and stacks the adenine ring of ATP is shown in stick representation, like the two ATP molecules trapped at the NBD interface, and colored by elements (C, *green*; O, *red*; N, *blue*; and P, *orange*). An ATPase inactive mutant was used here (Glu171Gln) allowing the stabilization of this transient ATP-bound conformation. This figure was made with PyMOL using the PDB code 1L2T [65]

In ABC transporters with NBDs bearing consensual signatures motifs, the Walker B motif is immediately followed by an invariant Glu residue whose position in the three-dimensional (3D) structure is reminiscent of the catalytic Glu found in other ATPases [53, 56, 57]. The involvement of this residue as a catalytic base, as initially proposed based on mutagenesis and kinetic studies [58], has been later substantiated by the 3D structure of the maltose transporter [59], and this seemed to nail down the original controversy about this residue [60]. Additional motifs are present in the RecA-like subdomain including: (i) The Q-loop, a stretch of ~ eight amino acids starting with a conserved Gln and joining the RecA-like and α-helical subdomains, makes part of the interface with the TMDs. A conformational switch of the Gln residue during the

catalytic cycle, engaging the MgATP and moving away after ATP hydrolysis, may be involved in transmitting conformational changes between NBDs and TMDs. (ii) The H-loop contains a conserved His that acts as a linchpin in ATP hydrolysis by interacting with the γ-phosphate of ATP and the catalytic Glu [61]. (iii) The D-loop carries usually the conserved sequence "SALD," a distinctive feature of ABC proteins located downstream of the Walker B motif. When the NBDs are sufficiently close to each other, the D-loop establishes a complex hydrogen bond and electrostatic network with the Walker A motif and H-loop of the opposite NBD. Because of its position at the dimer interface, the "D-loop" originally referred to "dimer" [62], while it was later alluded to the invariant aspartic acid of the motif. In the NBD dimers, the D-loops also connect and stabilize the catalytic Glu and attacking water [59, 63]. By contacting the ATP-binding sites both in *cis* and in *trans*, the D-loops are likely to play a major role in the communication between the active sites, the control of ATP hydrolysis, and also the directionality and energy of the transport as shown recently [64].

The two other subdomains are specific to the ABC family. One is the α-helical subdomain, which contains the family signature motif. Its sequence usually starts with LSGGQ and belongs to a loop located at the N-terminus of an α-helix. The role of this motif had remained elusive for a long time until the 3D structures of ABC dimers were solved [62, 65]. Hence, in all ABC family members, the NBDs associate transiently in a head-to-tail dimer in which the ATP molecules are sandwiched between the Walker A motif of one domain and the signature motif of the other domain. The LSGGQ sequence makes extensive hydrogen bonds with the ATP and is required for ATP hydrolysis. The role of this motif is likely similar to the arginine finger present in some P-loop GTPases, which stabilize the active site of the opposite domain (see Fig. 4.1). Another motif present in this subdomain is the x-loop, defined as TEVGERG sequence in Sav1866 (see below). It is only present in exporters and precedes the signature motif in the α-helical subdomain [66]. Its name refers to the fact that it interacts with both intracellular loops. Based on its proximity with the signature motif, it has been hypothesized to transmit conformational changes between the ATP-binding site and the TMDs [67–70].

The other subdomain is called the β-subdomain. It encompasses the A-loop which is located upstream of the Walker A motif and bears a conserved aromatic (A) residue that stacks against the adenine ring of the nucleotide, helping to stabilize it [53, 71]. While providing extremely valuable insights into the mechanism of ABC transporters, the structures of isolated NBDs lead to flawed interpretations of catalytic mechanisms since the TMDs impose some structural constraints and alter the geometry of the catalytic sites [59, 72, 73].

4.4 Structures of Whole Drug Exporters

Crystallized exporters were captured in mainly two opposite conformational states: outward facing and inward facing (Fig. 4.2).

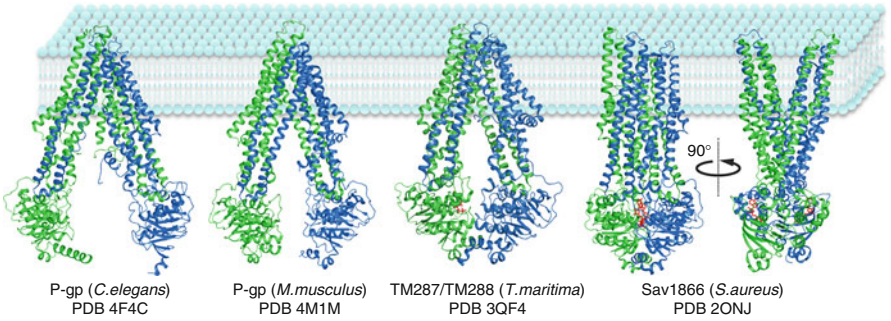

Fig. 4.2 3D structures of selected drug exporters from ABC family. The N-terminal half of P-glycoprotein (P-gp), TM287, and one monomer of Sav1866 are colored in *green*, while the C-terminal half of P-gp, TM288, and the other monomer of Sav1866 are shown in *blue*. When present, AMP-PNP is shown in *red stick* representation

Some structures originally contained major errors and were later corrected [74, 75]. Sav1866 is a multidrug transporter from *S. aureus* [76], and its crystal structure caused the retraction of erroneous MsbA structures [77] and thus revealed for the first time the correct architecture of an ABC exporter [66]. Sav1866 is a homodimer, and each protomer is made of six transmembrane helices located at the *N*-terminal side of the transporter and one *C*-terminal NBD (Fig. 4.2). Sav1866 was crystallized in an outward-facing conformation in which the NBDs are in close contact (closed state). Although this conformation was observed with ADP bound in the catalytic sites, this state most likely represents an ATP-bound state. Accordingly, a second structure with AMP-PNP bound instead was virtually identical [78]. The two monomers exhibit an extensive twist, and the domains of each monomer significantly contact those of the other monomer (Fig. 4.2). In this state, a central cavity was formed at the interface of the two TMDs. This cavity was shielded from the cytoplasm and the inner leaflet of the lipid bilayer, but accessible from the outer leaflet and the extracellular space (outward-facing conformation). The transmembrane helices are connected via short extracellular loops and long intracellular loops that protrude and extend the helical transmembrane bundles (Fig. 4.2). Consequently, the NBDs are located 25 Å away from the membrane. The TMD interface with the NBDs mostly involves the so-called coupling helices of the intracellular domains ICD1 and ICD2. The coupling helix 1 of ICD1 is located between transmembrane helices 2 and 3 and interacts mostly with the NBD of its own monomer. The coupling helix 2 of ICD2 located between transmembrane helices 4 and 5 interacts only with the opposite monomer (Fig. 4.3). This *trans* interaction of ICD2 is the trademark of the ABC exporters. Yet, the coupling helix 2 is rather similar to the coupling helix of the importers since it docks into a groove at the interface between the RecA-like and α-helical subdomains of the NBDs (Fig. 4.3). In this

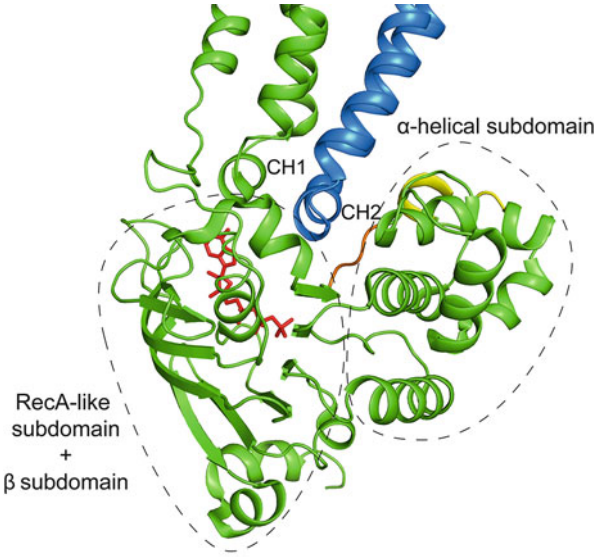

Fig. 4.3 Close view of the interface NBD/TMD in Sav1866 (PDB code 2ONJ). One monomer is shown in *green*, while transmembrane helices 4–5 and coupling helix 2 (CH2) from the other monomer are colored in *blue*. The Q-loop is colored in *orange*, while the x-loop is shown in *yellow*. AMP-PNP is visible in *red* sticks

closed outward-facing conformation, the interaction of the two NBDs is similar to the transient head-to-tail conformation of isolated NBDs.

MsbA is a lipid A flippase [79], but it also has the ability to transport some drugs [80, 81]. Depending on its origin and the crystallization conditions, three different conformations were obtained for this exporter: one which is very similar to the Sav1866 structure (closed AMP-PNP bound or ADP-Vi state, MsbA of *Salmonella enterica* serovar Typhimurium), one where the two NBDs are close to each other but not yet engaged in a tight interaction (closed apo state, MsbA of *Vibrio cholerae*), and finally one with the two NBDs widely separated in the so-called open state with an inward-facing conformation (MsbA of *E. coli* [EcMsbA]) [75]. In all the conformations, the transmembrane helices 4–5 and the associated coupling helix 2 cross over the homodimer interface and contact the opposite subunit. However, intracellular loop 1 loses contact with the opposite subunit in the open apo state. The open conformation has been subjected to controversy, but similar conformations were obtained for the mouse or *Caenorhabditis elegans* P-glycoproteins [82, 83], yet not as widely open as in the structure of EcMsbA (i.e., the NBDs are separated by ~ 25–30 Å in the P-glycoprotein *vs.* ~ 50 Å in EcMsbA). Interestingly, a structure of a flippase was recently solved in three different conformations: two open with various separations of the NBDs (44 Å and 30 Å) and one closed in an ADP-bound outward-occluded conformation [84]. Again, the two open inward-facing structures were suspected to be biased by the presence of detergent or crystal lattice contacts. However, the 3D structure of BmrA obtained in a lipidic environment was consistent with the open structures of P-glycoproteins [85], and this suggests that the presence of detergent in the X-ray crystallography experiments was not forcing the structure of the transporters in abnormal conformations.

The structure of an antimicrobial peptide exporter, McjD, offers presumably the first view of an intermediate conformation of the catalytic cycle of exporters, in an outward-occluded state [86]. While the two NBDs of McjD are still engaged in an ATP-bound conformation similar to that found in Sav1866, the TMD moiety shows a different organization of the transmembrane helices. This creates an internal cavity not accessible to either side of the membrane and that could possibly accommodate the transported molecule, i.e., the microcin J25.

The first structure of a heterodimer was obtained for TM287/TM288 [41]. It showed an inward-facing conformation at the membrane level, but the NBDs were only partially disengaged with significant contact being maintained at the degenerate ATP-binding site where an AMP-PNP molecule was still bound. However, even in the apo form of TM287/TM288, its two NBDs keep the same interaction/orientation at the degenerate site [87]. Hence, the authors raised the possibility that AMP-PNP is a poor ATP analogue for heterodimeric ABC transporters. Of note, AMP-PNP and AMP-PCP also failed to generate a closed NBD conformation in the homodimer of ABCB10 [88].

In addition to the crystal structures, the structure of the heterodimer TmrAB in a nucleotide-free state was obtained at a subnanometric scale by cryo-EM. It revealed an inward-facing conformation, yet a contact was maintained between the two NBDs at the level of the two *C*t-helices, one in each NBD [89]. Therefore, it is possible that a full physical separation between the two NBDs, in the nucleotide-free state, is a prerogative of homodimers or full-length transporters bearing two consensual ATP-binding sites.

4.5 An Alternating Access Mechanism

An alternating access mechanism seems the prevailing process in ABC transporters. It involves switching between two opposite conformations in which the substrate-binding site is alternatively accessible to one side of the membrane [75, 90–92]. Substrate binding on the inner or outer membrane leaflet and release on the opposite side are coordinated by the catalytic events, i.e., ATP binding, hydrolysis, and product release. Several studies on drug transporters suggest a lower affinity for drugs in the outward-facing conformation thereby explaining their release outside the cell [7, 93, 94]. The different conformational structures of MsbA lead to a transition model [75] in which pivoting of transmembrane helices 4–5 around the extracellular loops 2 and 3 brings the NBDs near each other; in this configuration, the NBDs are not properly aligned since the two Walker A motifs are facing each other, and a sliding movement of the NBDs along the interface would be required to align each Walker A motif with each LSGGQ motif, thereby pulling transmembrane helices 3–6 away from transmembrane helices 1–2. The newly formed outward opening is created between transmembrane helices 1 and 3, whereas the inward opening was formed between transmembrane helices 4 and 6. Recent molecular dynamic studies suggest twisting of the NBDs during the catalytic cycle of MsbA [95] and P-glycoprotein

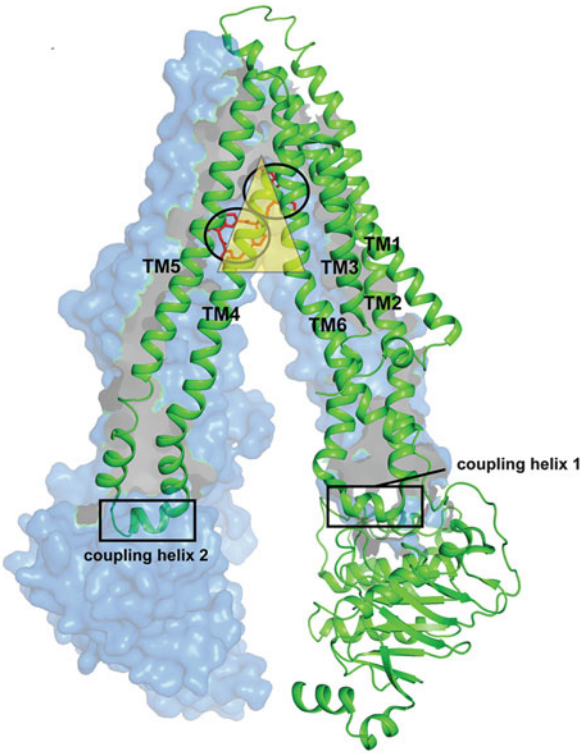

Fig. 4.4 Structure of P-glycoprotein in complex with two QZ59-SSS molecules (PDB 4M2T). The N-terminal half of P-glycoprotein is shown in *green* cartoon representation, and the C-terminal half is shown in *transparent blue* surface (*TM* transmembrane helix). The two drugs are colored in *red* and *circled* by *black spheres*. The putative drug-binding cavity is indicated with the *yellow triangle*

[96]. Furthermore, a misalignment of the NBDs was also observed in the crystal structures of ABCB10 [88], a putative transporter of heme precursors [97, 98]. In contrast, the NBDs in the heterodimer TM287/TM288 are partially interacting and are correctly aligned for canonical dimer formation [87]. Further experimental validation will be required to determine whether these conformational differences are physiologically relevant and truly reflect mechanistic differences between transporters. A variation of the classical alternating access mechanism has been recently proposed for the lipid-linked oligosaccharide flippase PglK of *Campylobacter jejuni* [84]. In this model, although the transporter can adopt inward- and outward-facing conformations, the substrate directly binds the outward-facing state and is flipped upon ATP hydrolysis.

4.6 Drug-Binding Sites

The most remarkable feature of MDR pumps is their ability to transport a wide variety of structurally dissimilar drugs. X-ray structures of murine P-glycoprotein revealed a large internal cavity open to both the cytoplasm and the membrane inner

leaflet, with a wide separation between the two NBDs [74, 82]. This configuration generates the presence of two portals at the level of the inner membrane leaflet. The first is located between transmembrane helices 4 and 6 on one side and the second between transmembrane helices 10 and 12 on the other side (Fig. 4.4). Of note, an *N*-terminal helical hairpin occludes one of these portals in the crystal structure of the *C. elegans* P-glycoprotein, but the overall shape of the two proteins is otherwise similar [83]. Drugs could reach access to the transport pathway from the aqueous phase [99–101] or through these portals within the membrane. Because many drug substrates partition and concentrate in the membranes [102], it is likely that drugs usually enter the transporter through the membrane inner leaflet. Consistent with this, Jin et al. observed a 100- to 4,000-fold increase in drug apparent affinity when studying the drug-stimulated ATPase activity of P-glycoprotein in membranes as compared to detergent [83].

The identification of the drug-binding site(s) in P-glycoprotein has been the goal of many studies (see a review in [103]). It was early recognized that a drug-binding site was localized within the TMDs [104]. Binding and kinetic analysis suggested the presence of several drug-binding sites [105–107]. Based on kinetic studies, Shapiro and collaborators proposed the existence of three drug-binding sites in P-glycoprotein, named H (Hoechst), R (rhodamine), and P (prazosin and progesterone) sites [108, 109]. Both the H and R sites are competent for transport, while the P site is an allosteric site. The R site preferentially binds rhodamine 123 and anthracyclines; the H site preferentially binds Hoechst 33342, quercetin, and colchicine; the P site binds preferentially prazosin and progesterone. The existence of two different H and R sites in P-glycoprotein was also evidenced by Förster resonance energy transfer (FRET) studies from Sharom's laboratory [110, 111]. A positive cooperative effect between the R and H sites was observed: the addition of a small concentration of a drug that binds to one site stimulates the transport of the substrate bound to the other site. Such reciprocal drug transport stimulation was also later observed with LmrA [7] and BmrA [11]. Shapiro and Ling also reported that other drugs such as vinblastine, etoposide, and actinomycin D compete with both H and R sites. Cysteine-scanning mutagenesis and thiol-reactive drugs such as dibromobimane, methanethiosulfonate-rhodamine, and methanethiosulfonate-verapamil were extensively employed to localize drug-binding sites in P-glycoprotein [112]. A common drug-binding pocket was found at the interface between the TMDs that can accommodate at least two drugs [113].

Murine P-glycoprotein was co-crystallized with two stereoisomers of cyclic hexapeptide inhibitors, cyclic-tris-(R)-valineselenazole (QZ59-RRR) and cyclic-tris-(S)-valineselenazole (QZ59-SSS). Either one molecule of QZ59-RRR or two molecules of QZ59-SSS were found in the central cavity of P-glycoprotein (Fig. 4.4) [74, 82].

The drug-binding cavity contains nine aromatic residues that are identical in human and murine P-glycoprotein but not conserved in *C. elegans* P-glycoprotein. There are no charged residues in the drug-binding pocket of mammalian P-glycoprotein structures, in contrast to *C. elegans* P-glycoprotein and MsbA, which has 16 charged residues pointing directly toward the substrate translocation

pathway [74]. Knowing the position of the two cyclic peptides QZ59-RRR and QZ59-SSS in the central cavity of the mouse P-glycoprotein, Martinez et al. sought to localize the H and R sites by assessing whether these peptides compete with the transport of Hoechst 33342 and daunorubicin and by performing molecular docking simulations [114]. They proposed the location of the H site along the central cavity and the QZ59-SSS molecule closer to the center of the membrane, with the R site at a deeper position in the cavity, overlapping the location of the QZ59-SSS molecule most embedded in the structure (see also [103]). Another group proposed similar locations for H and R sites, and the potent inhibitors tariquidar and elacridar bind to P-glycoprotein sites that coincide or overlap with these sites [115]. Importantly, the suggested R site is consistent with the cross-linking studies with methanethiosulfonate-rhodamine [112]. However, daunorubicin-binding site in MsbA was mapped at a different location, closer to the inner leaflet of the membrane [116]. Since the physiological substrate of MsbA is lipid A, it is conceivable that drugs opportunistically accommodate to the binding pocket of the transporter, possibly in a different location from typical multidrug transporters [117].

4.7 Basal ATPase Activity in Multidrug Transporters

Multidrug transporters typically display a high basal ATPase activity, which for bacterial transporters is often moderately stimulated by drugs [11, 30, 118, 119]. This behavior contrasts for instance with the well-coupled peptide exporter complex TAP [120] or some ABC importers [121–123]. Nevertheless, several lines of evidence suggest that drugs binding facilitate the dimerization of the NBDs thereby stimulating ATP hydrolysis [124, 125]. These observations are reminiscent of the mechanism of ATPase stimulation in other ABC transporters by allocrites [126], partner proteins [127], or proteins delivering the solute to importers [128–130].

Several plausible explanations could account for this seemingly “futile” ATPase activity in drug transporters. First, it might be due to nonoptimal conditions of purification or reconstitution procedures; given that this behavior is widespread among differently purified multidrug transporters, this seems unlikely. Second, it might result from the transport of lipids that could stimulate the ATP hydrolysis of the transporters [42, 80, 131, 132]. Third, it might be an intrinsic property of drug transporters, as suggested by a thermodynamic analysis of P-glycoprotein activity [133]. Ernst, Schmitt, and collaborators have proposed an elegant hypothesis: the kinetic substrate selection model [134, 135]. The basal ATPase activity may have the advantage of maximizing the number of transporters competent for substrate binding in inward-facing conformations. This model proposes that the time spent in the inward- or outward-facing states affects substrate selection and explains how two substrates with identical affinities, but dissimilar k_{on} and k_{off}, can be transported with different efficiencies. MDR transporters have the unique ability to recognize a huge variety of structurally dissimilar substrates. If the ATPase activities of these transporters were tightly coupled to the drug extrusion process, then only the substrates

capable of stimulating the ATPase activity would be transported. Possibly, a tight coupling would only be achieved at the expense of substrate diversity. Being capable to switch rapidly between the two opposite conformations, inward facing (i.e., in a conformation allowing to capture a noxious compound if present) and outward facing in an ATPase active conformation, even in the absence of a drug, might be the price to pay to make sure that any bound drug will be rapidly expelled out of the cell before being released from the transporter in an on and off process. Given the apparently relatively low affinity for many drugs, a fast rate of ATPase activity (coupled or not with the transport process) might overcome the rate constant of the drug release (k_{off}). Thus, wasting some energy in the absence of a drug might ensure the polyspecificity for many unwanted molecules and their efficient efflux once captured by the transporter.

4.8 Transport Mechanisms and Structural Flexibility of Multidrug Transporters

During the catalytic cycle, the two NBDs of ABC transporters engage and disengage with each other [129, 136]. Because the two ATP-binding sites are localized at the interface of the two monomers, ATP binding promotes the formation of a closed conformation [137, 138]. Although the interface of dimerization was a matter of debate for some time [139], the head-to-tail orientation first envisioned by Jones and George [140] was observed in the crystal structure of the ABC protein Rad50 involved in DNA double-strand break repair [62]. Later, this arrangement was validated with the crystal structure of an NBD dimer stabilized by the mutation of the catalytic glutamate [65] and the photocleavage of both the Walker A and LSGGQ motifs by the transition state analogue orthovanadate in the maltose transporter [141]. The latter observation also indicated that ATP hydrolysis occurs only in the closed conformation. Consistent with this, mutations in the LSGGQ motif strongly alter the ATPase activity of ABC transporters [142, 143]. The release of Pi and/or ADP destabilizes the dimer such that the NBDs move apart from each other. In addition to the interdomain movement, the RecA-like and α-helical subdomains within each NBD rotate toward each other upon ATP binding and move outward in the post-hydrolysis stage [52, 139, 144]. Hence, the energy of ATP binding and hydrolysis is coupled to conformational changes in the TMD thereby mediating alternating access of the substrate-binding site to each side of the membrane.

Several inward-facing conformations of P-glycoprotein exhibiting different degrees of domain separation were crystallized [74, 83, 145] hence suggesting a highly flexible protein. The distance between the α-carbons of the Walker A cysteines in the mouse or *C. elegans* P-glycoprotein varied between 38 and 53 Å [103]. These observations are consistent with the flexibility reported in the apo states of LmrA [146] and BmrA [147] in detergent. This flexibility evidenced by high rates

of H/D exchange in the apo state of BmrA is presumably caused by multiple conformations of the two ICDs, thereby allowing some freedom of rotation of the NBD [147]. In line with this, Cys-Cys cross-links were obtained for BmrA between the NBD and ICD1 that suggests the existence of additional, possibly transient, conformations of BmrA in the resting state [148]. Additionally, it was possible to cross-link two Cys residues, one in each Walker A motif of the P-glycoprotein suggesting that the two NBDs adopt alternate orientations in the resting state [149]. P-Glycoprotein structures have been suspected to exhibit nonphysiological conformations due to the absence of lipid bilayer and nucleotides. However, Wen et al. recently showed that, in intact lipid bilayers and in the presence or absence of nucleotides, P-glycoprotein adopts a wider range of conformations (both longer and shorter) compared to the original mouse P-glycoprotein crystal structure [150]. The authors suggested that this flexibility might originate from a high number of Gly and Pro residues thereby causing kinking and/or unwinding within the TMDs. Such flexibility may be advantageous to accommodate substrates of various sizes and chemical properties.

The mode and extent of NBD separation in MDR transporters is, however, still under debate [151], and two main hypotheses describing the mechanism of action of ABC transporters have been proposed: the ATP switch model [152] and the constant contact model [153].

4.8.1 The ATP Switch Model

In the switch model [152], which is also referred as the processive clamp model [154], the NBDs are proposed to dimerize upon ATP binding, sequentially hydrolyze ATP, and completely separate upon release of Pi and/or ADP. The ATP-dependent dimerization generates the outward-facing state, during which the drugs are translocated from the inner to the outer membrane leaflet, while ATP hydrolysis and release of hydrolysis products reset the transporter to the inward-facing conformation. This model largely relies on the available structures of ABC transporters in nucleotide-free or nucleotide-bound conformations. Many experimental data support this model. ATP binding promotes association of isolated NBDs [155] and ATP hydrolysis induces their dissociation [156]. In the context of intact transporters, biophysical and cross-linking studies suggest large-scale movements in MsbA [136, 157–159]. Moreover, ATP binding promotes large conformational changes in LmrA [146] and BmrA [160]. The main concern regarding this model is that apo states, as studied in biochemical experiments, may not be physiologically relevant. Given the prevalence of the nucleotide in cells, it has been proposed that transporters will likely have ATP bound *in vivo* [161]. Yet, cells and microorganisms in particular have to face stressful conditions that will strongly deplete ATP concentrations (see the discussion in [85]). Moreover, even in optimal laboratory conditions and for fast-growing *E. coli* bacteria, the ATP level can vary greatly among a bacterial population that originates

from a single clone [162]. Two other points are worth considering. First, in which conformational state ADP is released from the transporter? If ADP is released in the open state, then the transporter will return to this state before ATP can bind again, regardless of its concentration in the cell. Second, it is not the ATP concentration itself that really matters but rather the k_{on} of ATP binding. If this rate is slow as compared to the rate of transition between the closed state (just after ATP hydrolysis) and the open state, then the transporter will be able to return to the open state before ATP binds again. Considering all these parameters, the apo state should not be so infrequent for multidrug transporters, in particular in bacteria. In order to test the presence of the inward-facing conformation of P-glycoprotein in mammalian cells, Loo and Clarke [163] placed reporter cysteines in extracellular loops close enough to form a disulfide bond in this conformation but widely separated in the outward-facing conformation. Spontaneous cross-linking strongly suggested the existence, at least transiently in cells, of the inward-facing conformation in which the NBDs are open.

4.8.2 The Constant Contact Model

An alternative model has been proposed by Jones and George [153], in which the NBDs remain in contact throughout the catalytic cycle. This model should not be mistaken with a constant peripheral interaction between NBDs, as, for instance, in many ABC importers. In this constant contact model, ATP hydrolysis occurs alternately at each site, with one site able to open and exchange hydrolysis products, while the other ATP-bound site remains closed. Hydrolysis of ATP promotes an opening at that site by an outward rotation of the RecA-like subdomain relative to the helical subdomain [164]. This model built on earlier P-glycoprotein work from Senior and collaborators [165]. They proposed an alternating hydrolysis of the NBDs based notably on the observation that both sites were equally active and that orthovanadate-induced ADP trapping in one catalytic site was sufficient to inhibit ATP hydrolysis in both sites [166]. The occlusion of one nucleotide during the transition state has indeed been observed in several proteins: P-glycoprotein [165], BmrA [58], LmrA [7], and maltose transporter [167]. In contrast, two nucleotides were shown trapped in the heterodimeric TmrAB transporter [18]. The asymmetry observed in structural [168], biochemical [169–171] and molecular dynamic studies [164, 172] is often interpreted in favor of the constant contact model.

Another argument cited in favor of this model is that P-glycoprotein retains an ATPase activity when the NBDs are covalently linked together [173–175]. A single molecule FRET analysis of reconstituted P-glycoprotein rather supports a model where the NBDs do not completely dissociate from one another during steady state catalysis although, given the broad distance distribution recorded in all ligand conditions, full dissociation of the NBDs cannot be entirely excluded and may occur during some of the cycles [176]. This model involves an alternating catalysis in which ATP hydrolysis and Pi release are coupled to drug transport.

Lastly, the NBDs in the crystal structures of TM287/TM288 remained in contact, but with coupling helices separated by 15 Å, which is sufficient to make the substrate-binding cavity accessible from inside without the need for NBD full disengagement [87].

4.9 Concluding Remarks

It should be noted that alternate models are rarely discussed but could be as plausible as the models discussed above. For instance, one can imagine a scenario in which the binding of two ATP molecules generate an outward-facing conformation, as in the switch model, but the hydrolysis of one ATP molecule is sufficient to destabilize the dimer thereby implying a catalytic asymmetry. Whether the two catalytic sites in homodimeric ABC exporters hydrolyze ATP simultaneously, sequentially, in an alternating or stochastic manner has not yet been settled. In the isolated NBDs, MJ0796, the hydrolysis of one molecule of ATP is sufficient to allow the physical disengagement of the two NBDs [156]. Although a stoichiometry of two ATP molecules per substrate transported has been found for the OpuA importer [177], a stoichiometry of one ATP molecule was determined for P-glycoprotein [178]. Owing to the structural and functional diversity of ABC transporters, there might not be a single unified mechanism for all members. For instance, one of two catalytic sites is poorly active in heterodimeric ABC exporters, and such transporters may employ a different catalytic cycle than the homodimeric transporters. Recently, Mchaourab and colleagues proposed that the power stroke for drug export by BmrC/BmrD is the ATP hydrolysis step, in contrast to homodimeric exporters like MsbA where the transport process will occur during the NBD dimerization driven by ATP binding [31].

The recent 3D structure of ABCG5/ABG8, the human sterol exporter exemplifies again the diversity of this family. It shows a unique structure with some traits similar to importers like the lack of cross talk afforded by a coupling helix [179].

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