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Insights & Perspectives

Flipping and other astonishing transporter dance moves in fungal drug resistance

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Abstract

In all domains of life, transmembrane proteins from the ATP-binding cassette (ABC) transporter family drive the translocation of diverse substances across lipid bilayers. In pathogenic fungi, the ABC transporters of the pleiotropic drug resistance (PDR) sub-family confer antibiotic resistance and so are of interest as therapeutic targets. They also drive the quest for understanding how ABC transporters can generally accommodate such a wide range of substrates. The Pdr5 transporter from baker's yeast is representative of the PDR group and, ever since its discovery more than 30 years ago, has been the subject of extensive functional analyses. A new perspective of these studies has been recently provided in the framework of the first electron cryo-microscopy structures of Pdr5, as well as emergent applications of machine learning in the field. Taken together, the old and the new developments have been used to propose a mechanism for the transport process in PDR proteins. This mechanism involves a "flippase" step that moves the substrates from one leaflet of the bilayer to the other, as a central element of cellular efflux.

KEYWORDS
ABC transporter, ATPase activity, fungal drug resistance, single particle cryo EM

INTRODUCTION

In recent years, the phenomenon of multidrug resistance (MDR) has become a matter of public interest and a focus of research as one of the major obstacles to efficacious cancer chemotherapy and treatment of infectious diseases.[1] WHO estimates that the number of deaths per year in 2050 due to infections will overtake those due to cancer and cardiovascular disease, the two main causes of deaths worldwide presently. One of the many mechanisms of MDR involves the activities of membrane transport proteins that expel cytotoxic compounds from the cell. In eukaryotes, a protein group involved in membrane-mediated MDR in eukaryotes is the family of ABC transporters.

In fungi, MDR is often referred to as pleiotropic drug resistance (PDR), and the transporters contributing to the phenotype as the PDR network,[2] which in baker's yeast is composed of at least eight ABC transporters.[3] The network is localized in the plasma membrane and forms the first line of defense of these single cell eukaryotes. Expression of the PDR network transporters is controlled by two Zn^{2+}-dependent transcription factors[4] (Pdr1 and Pdr3) that, upon binding of xenobiotics, increase the transporter expression levels. In *Saccharomyces cerevisiae*, Pdr5 is a key player of the PDR network. Since its discovery more than thirty years ago, Pdr5 has been found to export hundreds of structurally unrelated compounds.[5–8]

As is typical for ABC transporters, Pdr5 consists of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). For many other ABC transporters, the TMD and NBDs can be in separate or fused polypeptides, and for Pdr5 the four domains are arranged

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Abbreviations: ABC, ATP-binding cassette; IF, inward-facing; MDR, multidrug resistance; NBD, nucleotide-binding domain; NBS, nucleotide-binding site; OF, outward-facing; PDR, pleiotropic drug resistance; TMD, transmembrane domain; TMH, transmembrane helix

in one continuous, long polypeptide with a direct repeat of NBD-TMD "units." In those units, the NBDs of Pdr5 are N-terminal to the TMDs (denoted as (NBD-TMD)$_2$), and strikingly, this topology is "inverse" compared to most other members of the ABC family, for which the TMDs are N-terminal to the NBDs. Additionally, Pdr5 has a distinctive N-terminal extension of approximately 150 amino acids common to all fungal ABC transporters from the PDR subfamily. While the conservation implies biological importance, the function of this characteristic feature is unknown.[9] Pdr5 is also distinguished by multiple deviations from the canonical sequence motifs present in other NBDs. The deviations are localized in residues which are crucial for ATP hydrolysis within the Walker A and B motifs, the Q-loop and the C-loop, which together form the nucleotide binding site (NBS). Interestingly, the deviating amino acid residues still participate in ATP binding and hydrolysis in one of the two NBS, namely NBS2. However, in NBS1 the deviations render this part incapable of hydrolyzing ATP[10] and thus it is often described as "degenerate." For NBS1, the canonical lysine residue of the Walker A motif becomes cysteine, the glutamate residue of the Walker B motif becomes asparagine and the glutamine of the Q-loop becomes glutamate. In another conserved motif, the H-loop, the histidine becomes tyrosine in NBS1. As the hydrolysis mechanism requires dimerization of NBDs, the C-loop needed to complete the NBS in one NBD domain is contributed by the partner NBD. So, the C-loop of NBS1, which is present in the more conserved NBD2, is mutated accordingly, with sequence L**NEV**QR instead of the more canonical V**SGG**ER of NBD1. The two residues contacting the γ-phosphate moiety of the bound ATP in the NBS are highlighted in bold. This comparison clearly shows that the catalytically essential residues of NBS1 are not present, while in contrast, NBS2 harbors all the catalytically important residues and represents therefore the active or canonical side. Consequently, Pdr5 is often referred to as an asymmetric ABC transporter.[11] Despite its apparent lack of ATPase activity, the degenerated NBS1 has been found to be essential for the function of Pdr5 in mutational studies.[12] The presence of an active (canonical) and inactive (degenerated) NBS raises important mechanistical questions that will be introduced in the following sections.

SETTING THE STAGE WITH STRUCTURAL SUPPORT

While Pdr5 has been intensively studied in the past decades,[9] a high-resolution structure of this protein, or of any other member of the PDR subfamily, remained elusive for over 30 years. In part, progress was impeded by difficulties in obtaining stable Pdr5 preparations. However, recent improvements of the purification protocol led to highly homogenous, and functional Pdr5.[13] This breakthrough provided the base for extensive structural investigation. Additionally, the introduction of peptidisc for reconstitution of detergent-solubilized protein permitted the stabilization of Pdr5 in a native-like and detergent-free environment that preserves its ATPase activity. The short amphipathic bihelical peptides that form peptidiscs can be used as an alternative to detergents and are compatible with the structure determination by single-particle cryo-EM.[14]
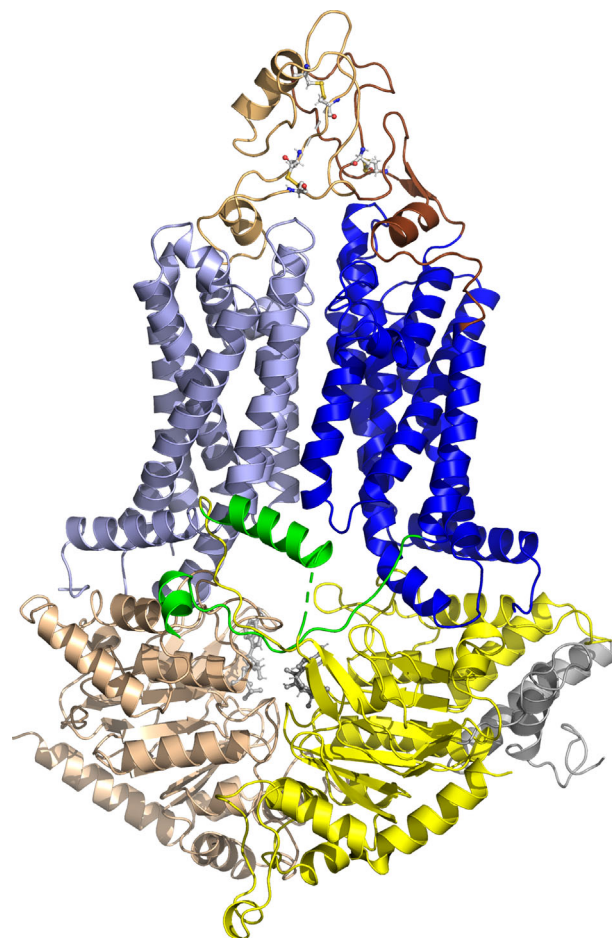
FIGURE 1 Single particle cryo-EM structure of ADP-Pdr5. Color-coding is as follows: N-terminal extension in gray, NBD1 in yellow, TMD1 in dark blue, linker domain in green, NBD2 in wheat, TMD2 in light blue, the extracellular domain 1 (ECD1) in brown and ECD2 in orange. The two disulfide bridges in the extracellular domain between residues 722 and 742 (ECD1) and 1441 and 1455 as well as 1427 and 1452 (ECD2) are shown in ball-and-sticks representation. The figure was prepared using PyMOL[19]

These improved preparations enabled the determination of the molecular structure of Pdr5 as the first PDR subfamily member, using single particle cryo-EM (Figure 1).[15] As expected, Pdr5 adopts the fold of type II exporters,[16] which in the newly introduced nomenclature has been renamed as type V fold.[17] Pdr5 structure was solved for four different functional states of the transporter using maps at resolutions ranging from 2.9 Å to 3.8 Å. This study revealed novel insights into the nucleotide-driven transport cycle and the relevance of a conserved linker domain. The obtained cryo-EM maps correspond to the following four different states of Pdr5: (i) apo-Pdr5 representing Pdr5 in the absence of nucleotides or substrates; (ii) ADP-Pdr5 representing the transporter with bound ATP in the degenerated site and ADP in the canonical site; (iii) R6G-Pdr5 representing the second state with the dye rhodamine 6G (R6G) bound in the transport channel; and (iv) AOV-Pdr5 representing the transporter with bound ATP in the degenerated site and ADP-VO4^{3-} in the canonical site. The latter trapped

the transporter in an outward-facing conformation by mimicking the posthydrolysis state.

The machine learning structure prediction program *AlphaFold*[18] models the structure of Pdr5 (https://alphafold.ebi.ac.uk/entry/P33302) with striking congruence to the experimental one, with a closer match to the outward facing state (1.7 Å root-mean-square deviation (rmsd) for overlay of peptide backbone) compared to the inward-facing state (2.3 Å rmsd). The greatest differences for both states are concentrated in the location of the N-terminal domain, which is likely flexibly connected with the NBD. These comparisons reflect on the powerful utility of machine learning predictions in providing accurate folds, but also its current limitations in predicting conformational switches.

A DISTINCTIVE ARCHITECTURE TO SUPPORT THE STAGE AND PERFORMANCE

The high-resolution maps reveal that Pdr5 adopts the architecture of an asymmetric, full-size ABC transporter with pseudodimeric NBDs as well as TMDs. Even though both NBDs of Pdr5 are structurally similar, multiple mutations in crucial amino acids are found in NBD1 (except for the C-loop of this NBD, which complements the conserved NBS2 in NBD2). Together with the substitutions in the C-loop of NBD2, the mutated residues form a degenerated NBS, which is unable to hydrolyze ATP and retains the unhydrolyzed nucleotide in all three studied non-apo states of the transporter. The more conserved NBD2 and the C-loop of NBD1 form a canonical NBS2 capable of hydrolyzing ATP, with hydrolyzed ADP present in two structures of Pdr5.

The substrate channel is located between the pseudo-dimeric TMDs, forming a large cleft stretching more than halfway through the cell membrane. The structure of Pdr5 in the presence of R6G reveals that the substrate R6G is located in the large cavity between TMD1 and TMD2, comparable to the location of substrates in other multidrug ABC transporters.[19–21] A structural comparison of Pdr5 with ABC transporters of the ABCG subfamily from human[16,22] and several bacteria[23,24] revealed that Pdr5 is structurally more related to the human ABCG transporter.

A closer look at the NBSs revealed a conserved linker domain located near NBS1 and contacting ATP (Figure 2). Sequence alignment of PDR transporters yielded a highly conserved sequence motif MQKGEIL found within the subfamily. In Pdr5, this distinctive feature consists of two stretches, LD1 and LD2, situated between the two halves of the transporter.

The first part—LD1—is formed by a loop extrusion of 30 amino acids between the first two ß-sheet strands of the degenerated NBD1 whereas the arch-shaped second part—LD2—connects the C-terminus of TMD1 with the N-terminus of NBD2. Fragments of both stretches directly contact the ATP binding site in NBD1, among them the highly conserved motif, [800]MQKGEIL[806]. In contrast, the linker domain appears to be highly conformationally variable in the apo-form, implying that it becomes ordered upon binding of ATP. Mutation of the linker motif has little impact on ATPase K_m values whereas the v_{max}

K[800]QKGEIL[806]
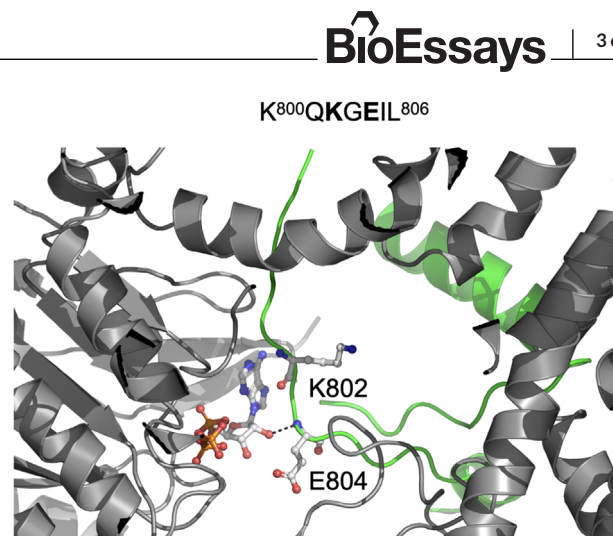
FIGURE 2 Details of the structure of the degenerated NBS1 in the ADP-Pdr5 state model. The linker domain is shown in green, interacting residues (K802 and E804) of the linker domain and the bound ATP molecule are highlighted in ball-and-sticks representation

values were highly reduced compared to the wild type, suggesting an allosteric effect of the linker domain on the enzymatic activity of Pdr5. In vivo, mutation of the linker motif residues increases sensitivity to certain drugs. This supports the proposal that Pdr5 is an "uncoupled transporter"[10] refering to the fact that ATPase activity is not stimulated by substrates, that is, that the hydrolytic activity is always present. Moreover, the data show that the highly conserved linker motif plays a crucial role in sensing ATP in the degenerated NBS1, as well as communicating with the canonical, active NBS2.

The two NBS of Pdr5 have other notable features. As pointed out above, NBS1 is degenerated and not capable of hydrolyzing ATP, while NBS2 is canonical (or active) and supplies the energy for substrate translocation. Analysis of the interactions of the bound nucleotides in the IF and OF state reveals interesting differences (Figure 3). In the ADP-Pdr5 structure, ADP is coordinated by backbone and side chain interactions with the Walker A motif, namely through residues Lys 911 and Thr 913 (Figure 3A). Interestingly, no aromatic A-loop residue is present in the vicinity, which represents a clear deviation from the classic coordination of the adenine moiety of the bound nucleotide. In the AOV-Pdr5 structure (Figure 3B), the catalytic Glu 1036 participates in binding to the ADP-V_i bound nucleotide. For the degenerated NBS (NBS1), the bound ATP is coordinated by residues of the Walker A motif and the linker domain (Figure 3C). Again, an aromatic A-loop residue is not present. Rather, the adenine moiety is bound in a hydrophobic, shallow cavity, similar to the canonical site. This might explain why Pdr5 can easily use other nucleotide triphosphates, such as GTP, as energy source.[25] The linker domain senses the bound ATP in the IF (ADP-Pdr5), as well as in the OF (AOV-Pdr5) state (Figure 3D), again indicating the sensory function of the linker domain. The only difference between both states is the coordinating amino acid residue. In the IF state, Glu 804 coordinates the nucleotide, while in the OF state it is Lys 802. As mentioned above, a mutational analysis of the linker domain residues that sense the bound nucleotide in the degenerated NBS highlighted its important functional impact.
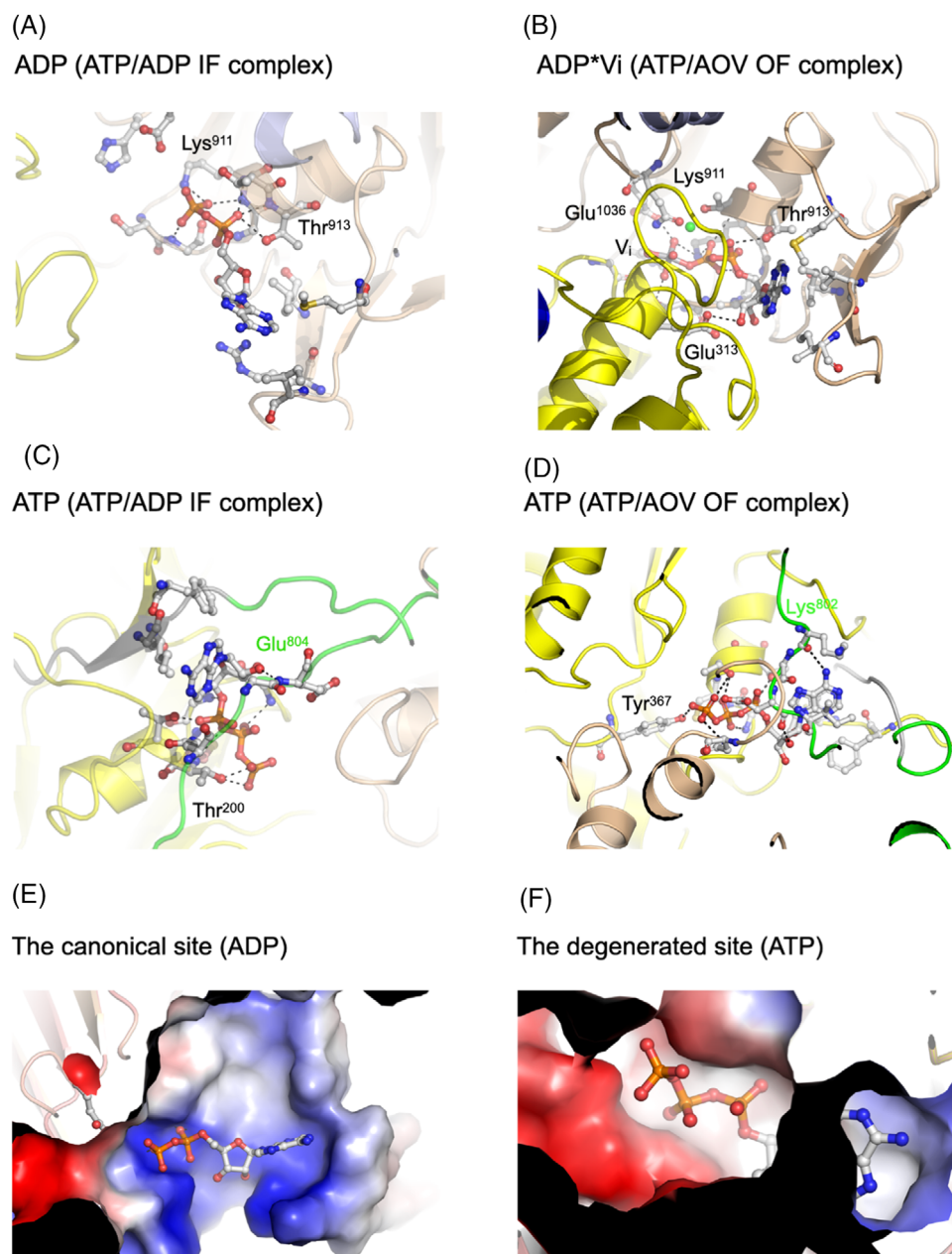
FIGURE 3 Zoom-in into the NBS of the canonical NBS in the ADP-Pdr5 structure (A) and the AOV-Pdr5 structure (B). The degenerated NBS is shown in (C) for the ADP-Pdr5 structure and for the AOV-Pdr5 structure in (D). Electrostatic surface calculations of the canonical NBS2 of the ADP-Pdr5 structure (E) and the degenerated NBS1 in the ADP-Pdr5 structure (F). The bound nucleotide is shown in ball-and-sticks representation. Color-coding is as follows, blue: +5 kT, red: −5 kT. The electrostatic surface potential was calculated using the APBS electrostatics plugin in PyMOL[19]c

This suggests that the linker domain has not only a sensory, but also a structural role. As it is the first time that the linker domain of an ABC transporter was visualized in a structure model, structural information from other systems is needed to judge whether the role of the linker domain of Pdr5 is of general importance or is specific to this yeast ABC transporter only.

Another interesting aspect of the NBS is the electrostatic surface potential. In the canonical site, ADP is bound in a positively charged pocket (Figure 3E) compensating the negative charges of the diphosphate moiety. This is as expected and has been seen previously in other ABC transporters. However, surprisingly, the triphosphate moiety of ATP in the degenerated site is bound in a negatively charged pocket (Figure 3F). This obviously will result in charge repulsion and destabilize bound ATP. Further studies are clearly required to understand the functional role of this unexpected charge repulsion.

Comparison of the inward-facing (IF) and outward-facing (OF) structures of Pdr5 revealed an asymmetric motion of both NBD: specifically, very little motion in NBD1 but a high degree of movement in NBD2 upon switching the conformation between IF and OF (Figure 4).[15]
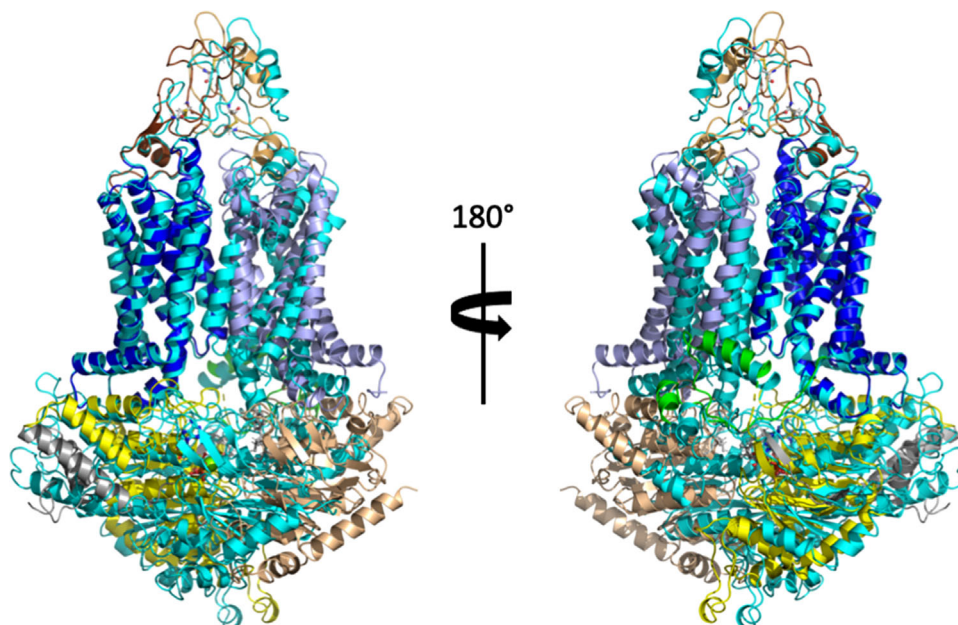
FIGURE 4 Superimposition of the ADP-Pdr5 structure (IF) (colored as in Figure 1) and the AOV-Pdr5 structure (OF) (colored in cyan). The comparison shows how only one half of the transporter moves during the IF–OF conformational switch

ORCHESTRATION OF THE TMDs

The asymmetry of motion of the NBDs is also mirrored in TMDs of Pdr5, with substantially larger structural changes in TMD2 compared to TMD1. In the resting state (IF), represented by ADP-Pdr5, the TMDs tilt toward each other on the extracellular side and spread further apart on the cytosolic face. This architecture blocks the exit channel and opens an entry cavity accessible from the cytosol as well as the inner leaflet of the membrane. As is typical for ABC transporters, each TMD of Pdr5 consists of six transmembrane helices (TMH). Whereas the first helix of each TMD (TMH1 and TMH7) lies parallel to the membrane surface, the other five helices span the lipid bilayer. It is interesting to note that TMH5 is split in three parts, as already seen in the structures of the two human ABCG transporters, ABCG2 and ABCG5/G8.[16,22] Here, TMH5b is nearly perpendicular to the direction of other helices.

The architectures of all different states that were obtained by single particle cryo-EM lends insight into the transport cycle of this ABC transporter. It was proposed that the ADP-Pdr5 structure defines the resting state, with an IF conformation, one ATP bound in the degenerated site, and one ADP in the canonical site. In this structure, the entrance of the substrate cavity between the two TMDs remains open. Whenever substrate is present, it will bind in the cavity. As is indicated by the R6G-Pdr5 model, the transporter remains in an inward-facing conformation with the substrate nestled between TMDs, and ADP remains in the canonical site. Only the release of ADP and binding of ATP to NBS2 induce a conformational change opening the exit channel to the extracellular space, as can be seen in the vanadate-trapped AOV-Pdr5 structure. This fact is also supported by various studies analyzing the structural changes of the transition state compared to the pre-hydrolytic state of ABC transporters.[26]

The change to an OF conformation enables the passage of the substrate through the substrate cavity in the direction of the now-open exit channel, releasing the substrate into the extracellular space or outer leaflet of the membrane. This is akin to a peristaltic pump motion,[15] and was also observed in ABCG2.[21] The hydrolysis of ATP induces a conformational shift back to the IF conformation with ADP remaining bound to the canonical site. In this state the transport cycle is completed and the transporter is ready to take up new substrate.

SUBSTRATE TAKES CENTER STAGE

In Pdr5, the substrate is localized in a large cavity between TMDs, comparable to the location of other substrates in the ABC transporters from the MDR family. Based on the R6G-Pdr5 structure, the helices forming the cavity are mainly TH1b, TH2, TH4, and TH5a of TMD1, as well as TH8 and TH11a of TMD2 (Figure 5). Roughly 40% of the residues of the helices surrounding the substrate R6G are hydrophobic amino acids leucine and phenylalanine. The phenylalanine residues form a ladder that appears to guide the substrate in the direction of the exit channel, which is topped by the extracellular domains (ECD1 and ECD2).

Several studies have investigated the substrate preferences of Pdr5,[27] and have noted that although most of the known substrates are hydrophobic, the volume rather than the hydrophobicity are important predictors for binding strength of the molecules. Substrates with a surface volume of 200–300 Å^3 were correlated with a high substrate binding strength, while a surface volume of under 90 Å^3 only had little impact on drug binding and transport. Nevertheless, the lipophilic character of the known substrates is in line with the chemical properties of the residues located in the large substrate cavity, since
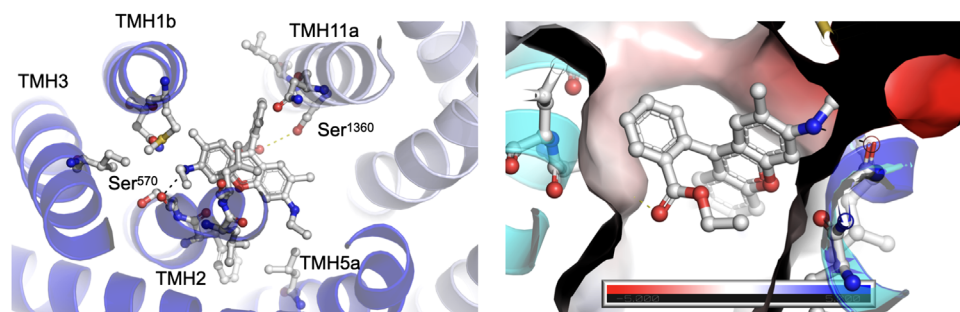
FIGURE 5 Zoom-in into the R6G binding site (left panel) and electrostatic surface potential (right panel). Color coding in the left panel is as in Figure 1. Only residues of Pdr5 that form hydrogen bonds with the bound substrate are displayed (in ball-and-sticks representation). Ser 1360 had been already identified biochemically.[42,43] The S1360F mutant, for example displayed a reduced R6G efflux and equally important did not show inhibition by FK506. In a subsequent study,[44] it was shown that FK506 is a competitive inhibitor of R6G demonstrating that both compounds bind to the same binding site in Pdr5. The bar in the right panel is color coded for electrostatic representation, as also shown in Figure 3. For a complete description of the binding site, including all the hydrophobic interactions, see Harris et al.[15]

approximately 65% of the residues have a hydrophobic character. Taking into account the surface volume of substrates and the amount of hydrophobic residues in the cavity, Pdr5 acts somewhat like a vacuum cleaner[28] drawing in all lipophilic substrates of appropriate size. Larger surface is synonymous with more interaction with residues in the cavity and therefore provides a higher binding strength. The location of R6G with the binding cavity coincides with the position of substrates visualized in the binding sites of ABCG2[21,29–31] (see Harris *et al.*[15]).

CONSERVATION OF THE NBDs AND IMPLICATIONS FOR THE PERFORMANCE

As pointed out above, Pdr5 is an asymmetric ABC transporter composed of an active (or canonical) nucleotide-binding site NBS1 and an inactive (or degenerated) NBS2. Analysis with *ConSurf*[32] revealed that the conservation of NBS1, the inactive site, is substantially lower than the conservation of NBS2 (Figure 6).

A high level of conservation is expected for the NBDs of ABC transporters, because the domains can be regarded as the power plant driving substrate transport. However, in Pdr5 the conservation is asymmetric, with a much higher level of conservation in NBD2 (wheat colored structure cartoon in Figure 6). NBD2 harbors most of the residues forming the active NBS2. This asymmetry suggests that the conservation of NBD2 is due to its function as the power plant, while the degeneration in NBS1, mostly within NBD1 (yellow cartoon in Figure 6), allows for more flexibility of the primary structure.

SUBSTRATE SOMERSAULT

How Pdr5 substrates reach their binding site in the IF state of MDR or PDR ABC transporters is still an open question. In principle, direct access from the cytosol, or alternatively, from the inner leaflet of the plasma membrane, can be envisioned.
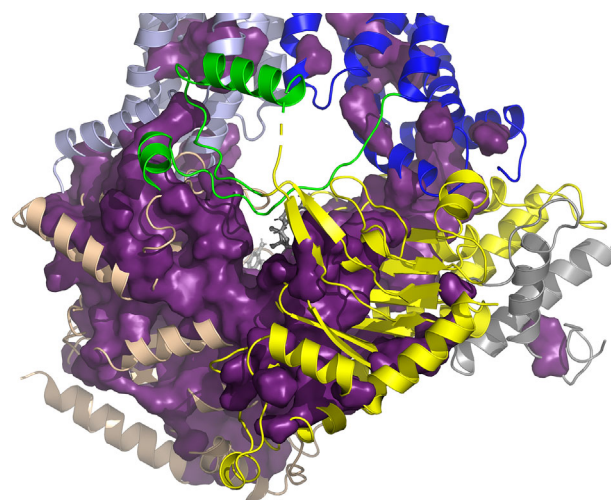
FIGURE 6 Zoom-in into the NBD regions of the ADP-Pdr5 structure. The N-terminal extension is shown in gray, NBD1 in yellow and NBD2 in wheat cartoon representation. The amino acid conservation is derived from 250 sequences obtained by blastp and calculated by ConSurf.[33] Only the amino acids with the highest conservation score are shown as magenta surface

An analysis of putative channels or entrance gates of Pdr5 using *Caver*[33] revealed a high degree of asymmetry, mirroring the observation for NBDs. As already shown in detail in Harris *et al.*[15], only one suitable entrance pathway exists in Pdr5. Interestingly, the pathway is situated at the interface of the cytosol and inner leaflet of the plasma membrane.

The location of the entrance pathway strongly suggests that substrates such as R6G bind to Pdr5 from the inner leaflet of the membrane, and not from the cytosol. This also implies that cytotoxic compounds will not reach the cytosol, as Pdr5 binds substrates directly from the inner leaflet preventing their migration into the cell interior. Considering the hydrophobic nature of the substrates of Pdr5, such uptake mechanism would ensure a highly effective protection.

The extracellular gate or exit pathway is asymmetric, as only one exit pathway is present in Pdr5.[15] This has been proposed earlier for other asymmetric ABC transporters[11] and seems to be a general issue of degenerated ABC transporters. Considering the proposed efflux mechanism, it is also important to stress that the ECDs of Pdr5 do not move substantially during the IF to OF switch, and crucially, an exit pathway is not specifically created by the movement (Figure 4). Rather, the switch opens an internal gate located at the interface of the outer leaflet of the plasma membrane and the extracellular space. In direct correspondence to substrate uptake mechanism described above, such architecture suggests that substrates are not released into the extracellular medium. Instead, they are likely released into the outer leaflet, which is thermodynamically the most preferred location for hydrophobic compounds compared to the hydrophilic environment of the extracellular space. This hypothesis also implies that Pdr5 acts as a flippase taking up substrates from the inner leaflet and releasing substrates to the outer leaflet of the plasma membrane of yeast.

This concept was already proposed for ABCB1 in 1992,[34] but it has not been empirically verified for any MDR or PDR ABC transporter. In the case of Pdr5, one could envisage for this purpose an experiment employing a fluorescent substrate that is quenched if it resides only in the membrane. To achieve this, first and foremost, an in vitro transport assay for purified Pdr5 needs to be established, and this has not yet been reported. Secondly, the quencher for this reaction would have to be *exclusively* present in the membrane. To ensure this, the concept might be exploited of doping cell membranes with lipids that bear a covalently attached fluorophore, such as NBD (*N*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-C_{16} lipids.[35] If the timing is right, the quencher-labeled lipid will only reside in the membrane and quench the substrate of Pdr5 while it is transported from the outer to the inner leaflet of the membrane and spontaneously flips back to the outer leaflet. Only if the substrate leaves the membrane, fluorescence will be detected. However, this also requires measuring the kinetics of substrate transport. Here, single-molecule techniques should be employed, to obtain the required spatial and temporal resolution in the presence and absence of the quencher lipid. Single-molecule approaches have been already used to investigate ABC transporters,[36–41] but not to analyze the transport mechanism of MDR or PDR transporters; only the structural re-arrangements of the NBDs of ABCB1 were analyzed.[40] Nevertheless, the described experimental set-up is obviously everything but straightforward, and so it might take time until a conclusive experimental proof of a possible flippase activity of Pdr5 is provided.

It is worth restating here that the proposal that Pdr5 acts as a drug flippase is different to the mode of action of ABCG2: the main entrance pathway of ABCG2 in inward facing state leads from the cytosol with only a minor entrance available from the water-lipid interface side. In the outward facing state, however, there is no obvious pathway into the outer leaflet and consequently the substrate is released into the extracellular medium.[21]

CONCLUSIONS

Over the last three decades, Pdr5 has become a model system to study the phenomenon of fungal MDR. More than 600 substrates for Pdr5 are known to date, but the principles of how the transporter can recognize and efficiently remove from the cell such a variety of chemically and structurally unrelated compounds is still not understood on the molecular level. Only recently, a protocol to purify Pdr5 to homogeneity in a functional state was established that enabled the first experimental structures of Pdr5 by single particle cryo-EM. The four structures obtained have provided important new insight into recognition of transport substrates and the series of conformational changes in this asymmetric ABC transporter that drive transmembrane transport. These include highly asymmetric motions during the IF-to-OF switch, the identification of a nucleotide sensor located in the linker domain, and the architecture of the degenerated NBS. Most importantly, based on a critical re-evaluation of these results, we propose that Pdr5 is a substrate flippase that shuttles its substrates from the inner to the outer leaflet of the plasma membrane of yeast. These data have provided a glimpse of the hitherto unseen movements in the orchestrated dance of nucleotide binding, hydrolysis, conformational state changes and directional movement of molecules that underpins the biological performance of this fascinating nanomachine.

CONFLICT OF INTEREST

The authors do not have a conflict of interest.

DATA AVAILABILITY STATEMENT

All data are available upon request from the corresponding authors.

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