Recent Advances in Understanding the Structure and Function Relationship of Multidrug Resistance-Linked ABC Transporter P-glycoprotein

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Abstract: Mammalian P-glycoproteins (P-gp) are members of the broad family of ABC transporters and play important physiological roles in establishing physical barriers that limit access of toxic compounds and thus in the pharmacokinetics of these compounds. Cancer cells exploit the presence of P-gp to fend off anti-cancer drugs, rendering them multidrug resistant (MDR). Structural investigations of P-gp involve the expression and isolation of this large integral membrane protein in high quality and in sufficient quantity for it to be amenable to electron microscopic (EM) and crystallographic studies. EM studies have defined the shape of the molecule and delineated its various conformations in solution but major breakthrough in obtaining atomic resolution structures of P-gp were accomplished by X-ray crystallography. Structures with increasing resolution and accuracy in various substrate and inhibitor bound forms are available for analysis and novel mechanistic insights have been obtained. These advances have paved the way for future research to further our understanding of the mechanism of P-gp function and development of potential inhibitors that may reverse MDR in cancer treatment.

Keywords: P-glycoprotein, ABC transporters, Multidrug Resistance, Mechanisms, Structures.

A prime difficulty in effective treatment of cancer by chemotherapy is the simultaneous development of cellular resistance to multiple anticancer drugs, known as multidrug resistance (MDR). MDR also remains a significant problem in treatment of microbial infections. One mechanism of MDR is the overexpression of ATP-dependent efflux pumps, represented by ABC transporters such as ABCB1 or P-glycoprotein (P-gp). MDR conferred by the ABC family of efflux transporters is achieved by pumping a wide range of different drugs out of cancer cells, thus reducing their effective intracellular concentrations for cytotoxicity [1]. Importantly, P-gp expression correlates with poor clinical response to chemotherapy in patients [2]. Complicating the issue, P-gp also plays an important physiological role in protecting tissues from potential toxins by being present in numerous physiological barriers such as the endothelial cells including the gastrointestinal tract, the blood-brain barrier, and the placenta-blood barrier. Understanding how P-gp recognizes and transports a wide variety of chemically unrelated compounds, how it couples the energy of ATP hydrolysis to substrate translocation, and how its activity can be modulated requires an accurate and detailed knowledge of the structure of the protein at atomic resolution. Most importantly, understanding these questions will help to control the function of P-gp in a clinical setting for effective delivery of drugs into cancer cells by (1) rational development of novel, targeted therapeutics and (2) screening for drugs that bind to the active sites of P-gp. In this review, we attempt to provide an overview on the effort in the field to understand, both biochemically and structurally, the mechanism of function of mammalian P-glycoprotein.

CELLULAR FUNCTION OF P-GP

Soon after the first successful chemotherapy of human cancer there was the realization that drug resistance was going to be a major problem in cancer treatment [1, 3, 4]. The combinatorial approach employing multiple drugs with different modes of action apparently was not the solution, since cancers appeared able to develop resistance simultaneously to many different anti-cancer drugs, for which the term multidrug resistance (MDR) was coined. The hope to circumvent MDR in order to improve cancer therapy led to extensive investigations into the mechanisms of MDR and the search for potential reversing agents.

Multidrug resistant hamster ovary (CHO) cells were isolated as early as in 1968 and the MDR phenotype was demonstrated correlating to the reduced drug accumulation inside the cell [4]. By 1974, it was shown that MDR exhibited in CHO cells is energy dependent [5] and in 1976 MDR was found to correlate with the expression of a surface glycoprotein of CHO cells with an apparent molecular weight of 170 kDa, which was termed P-glycoprotein [6]. P-glycoprotein or P-gp was purified from plasma membrane vesicles of resistant

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CHO cells in 1979 and accounted for 3-4% of total membrane proteins [7].

The relevance of the expression of P-gp in clinical MDR models was also investigated, showing cross resistance of multiple drugs once a tumor was treated with any one drug [3]. In 1979, a MDR human cancer cell line was isolated showing high expression of a surface glycoprotein that made the resistant cell line prone to aggregation [8] and the expression of this surface glycoprotein was shown to be widespread in various cancer cell types [9]. Expectedly, P-gp expression was detected using P-gp-specific monoclonal antibody in two clinical samples from patients with ovarian cancer [10]. Most importantly, it was shown that the MDR phenotype in cancer cell lines can be reversed by verapamil, raising the hope that clinical MDR can be circumvented [11]. Indeed, in a clinical trial for patients with acute myeloid leukemia (AML), it was shown that patients treated with the MDR inhibitor cyclosporine A (CsA) plus chemotherapy displayed a significant survival advantage [12].

The function of P-gp does not limit to conferring MDR in cancer, as mRNA of P-gp was found in various normal tissue types in addition to cancers [13]. In particular, the P-gp gene is highly expressed in the adrenal gland and in the kidney, moderately expressed in the lung, liver, lower jejunum, colon, and rectum, and expressed at low levels in many other tissues. These observations were supported at protein level using a P-gp-specific monoclonal antibody MRK16 to detect high expression level of P-gp in the adrenal cortex and medulla of adults and in the renal tubules of the kidney [14]. Furthermore, P-gp was found abundantly expressed at some physiological barriers such as the placenta-blood [14] and the blood-brain barrier [15]. These results suggest that P-gp also plays a physiological role in normal tissues, which was speculated to be mainly cellular detoxification. Indeed, P-gp is a major factor in reducing the oral availability of amphipathic drugs such as Taxol, [16] and P-gp knockout mice were viable under normal conditions but displayed severe sensitivity to toxic environments [17].

BIOCHEMICAL CHARACTERIZATIONS OF P-GP

The hope that the activity of P-gp can be inhibited has been one of the driving forces for detailed biochemical characterizations of P-gp. By 1986, P-gp genes from human, mouse and Chinese hamster were cloned and their primary sequences obtained [18-20]. P-gp was shown to be a member of the ABC transporter family, featuring a long polypeptide chain (1280 amino acid residues for the human P-gp) with two homologous halves; each half consists of a transmembrane domain (TMD) and nucleotide-binding domain (NBD). The initial attempt to purify human P-gp was conducted using drug-selected cell lines by immunoaffinity chromatography in one step, resulting in a protein that is preserved in its ATPases activity [21]. Heterologous expression of P-gp in insect cells yielded membrane proteins that have no glycosylation but maintained drug binding activity [22]. Isolated membrane vesicles from drug resistant MDR cells, but not from sensitive cells, were shown to accumulate drugs in an ATP-dependent manner [23]. Of particular interest, partially purified P-gp was shown to possess drug-stimulated ATPase activity when reconstituted into lipid [24]. These early studies paved the way for subsequent, more detailed biochemical and functional studies of P-gp function, centering on the ATP hydrolysis cycle and drug-binding pocket.

Like many other mechanistic studies, high quality, isolated P-gp was required for establishing clean, *in vitro* assay systems. This led to the development of heterologous recombinant expression systems for either human (*h*P-gp) or mouse P-gp (*m*P-gp) and subsequent purification procedures [25, 26]. Purified P-gp proteins were used in a flurry of biochemical characterizations including *in vitro* reconstitution in lipids [27], drug-stimulated ATPase activity [24], and vanadate (Vi) promoted trapping of ADP [28]. The results of these experiments led to the conclusion that both ATP sites of P-gp are important for function; they interact with each other and most likely hydrolyze ATP in an alternate fashion [29]. With the development of these methods, factors that influence P-gp function can be studied. Lipid molecules, for example, appear to have significant influence on the function of the protein. By mass spectrometry experiments it was shown that P-gp binds negatively charged bulkier lipid such as cardiolipins with preference for shorter hydrocarbon chains over longer ones. Competition experiments show that P-gp can bind simultaneously to lipids and CsA [30]. Moreover, It was found that the apparent affinities of P-gp for anticancer drugs actinomycin D and paclitaxel are approximately 4,000 and 100 times higher, respectively, in the membrane bilayer than in detergent [31].

To explore the substrate-binding site or to identify which amino acid residues are interacting with P-gp substrates or inhibitors, multiple techniques were employed. One was to construct a Cys-less (cysteine

free) P-gp, into which single or multiple cysteine residues were re-introduced for covalent interaction with thio-reactive agents or substrates to identify drug-binding sites [32-36]. Another method was to use radioactive photo-affinity substrates to cross-link (non-specifically) P-gp, leading to the proposal of two or more drug-binding sites in P-gp [37, 38].

Based on these biochemical analyses, various mechanistic models have been proposed. These models can be grouped into the following categories. (1) For substrate entry into the transporter, there is a hydrophobic vacuum cleaner model [39-41], which suggests drug-pickup by P-gp is mediated by membrane bilayer due to differential partitioning of hydrophobic drugs. (2) How substrates interact with the drug-binding sites has been a subject of extensive studies. Using a photoactive substrate and an allosteric modulator, Dey and colleagues proposed the existence of two drug binding sites, thus, the two–site transport model (ON and OFF) [37]. Consistent with this model, labeling by photoaffinity analog of drug-substrate showed altered affinity for the drug in the Vi-trapped transition state: a surrogate assay to monitor conversion of high affinity ‘‘ON’’ site to a low affinity ‘‘OFF’’ site [42]. By introducing cysteine residues to the TMD of P-gp systematically in a cysteine-less background followed by binding of thioreactive substrates, Loo and Clarke found that for each substrate multiple TM helices are involved in binding and the protein undergoes conformational changes. Thus they proposed the Induced-fit model [35]. (3) To exit the transporter, drug substrates were proposed to undergo a rehydration process [43]. (4) The two NBDs hydrolyze ATP to provide a driving force for substrate translocation. An Alternate-Site Model was proposed to account for observed asymmetry in nucleotide binding of the two NBDs [29]. There is also a Constant Contact model that proposes a close proximity of the two NBDs during the ATP hydrolysis cycle because inside the cell ATP is abundant and also because closed NBDs were observed from studies of bacterial ABC transporters in the presence of nucleotides [44, 45]. (5) The mechanisms dealing with the coupling between ATP hydrolysis and drug translocation require establishment of the transport stoichiometry (the ratio between molecules of ATP hydrolyzed per drug transported), which has been reported to be 2 [46] with one ATP for drug transport and the second ATP for resetting the transporter [47]. Importantly, vanadate binding (transition state) led to a lowered affinity for drug binding [42]. A half-coupled mechanism was proposed to account for polyspecificity of substrate recognition for P-gp [48]. Considering the biochemical evidence of multiple sites for drug interaction, van Veen *et al*. proposed a two cylinder-engine model for the coupling [49]. ATP binding and hydrolysis were also considered as the switch to control substrate binding and release in the ATP switch model [50]. Clearly, these mechanisms capture certain aspects of the P-gp function but are in need of integration into a unifying mechanistic model, which requires the assistance of structural studies of P-gp.

STRUCTURAL STUDIES OF P-GP, A BRIEF OVERVIEW

Despite much progress that has been made in understanding mechanistic aspects of P-gp function by biochemical and biophysical studies, a number of important questions remain unanswered. There is overwhelming evidence that P-gp binds nucleotide molecule one at a time [51-53]. However, whether a nucleotide molecule enters NBD1 or NBD2 in a predefined sequence or in a random fashion has not been resolved. ATP hydrolysis in one NBD has been shown to be dependent on the other one to be active. However, the structural basis of this dependence is not clear at all because no nucleotide can bind simultaneously to both NBDs. Moreover, we have yet to explain the chemical basis for the substrate polyspecificity of P-gp and we are still puzzled by the phenomenon of the basal ATPase activity, ATP hydrolysis in the absence of substrates. The observation of substrate-stimulated ATPase activity suggests the existence of a communication mechanism between the drug-binding site and the NBDs; understanding its structural basis remains a challenge. Most intriguingly, the conformation spectrum of P-gp, induced by ATP binding, hydrolysis or substrate recruitment, and required to complete the P-gp function of substrate extrusion, remains elusive. To address these mechanistic questions, we must obtain a structural framework that details all available parts for the P-gp function.

Although X-ray crystallography remains the method of choice for determining membrane protein structures, as evidenced by the relatively large number of membrane protein structures in the protein databank (PDB) determined annually by this method compared to alternatives, obtaining diffracting quality P-gp crystals has proven to be challenging. Other experimental techniques began to make contributions to structural studies of P-gp, most notably by high-resolution electron microscopy (EM). EM studies of *h*P-gp at low resolution showed conformational

Table 1: Available Crystal Structures of P-Glycoproteins from Different Species

PDB	Organism	Ligand or inhibitor	Nucleotide	Reso [Å]	R_{free}	Principal Investigator	Date Deposit	Ref
3G5U	*M. musculus*	Hg^{2+}	None	3.80	0.347	Geoffrey Chang	2009-03-24	[61]
3G60	*M. musculus*	QZ59-RRR	None	4.40	0.365	Geoffrey Chang	2009-03-24	[61]
3G61	*M. musculus*	QZ59-SSS	None	4.35	0.356	Geoffrey Chang	2009-03-24	[61]
4F4C	*C. elegans*[b]	Model ligands[c]	None	3.40	0.283	Jue Chen	2012-09-26	[31]
4KSB	*M. musculus*	None	None	3.80	0.357	Geoffrey Chang	2013-07-31	[62]
4KSC	*M. musculus*	None	None	4.00	0.338	Geoffrey Chang	2013-07-31	[62]
4KSD	*M. musculus*	Nanobody Nb592	None	4.10	0.344	Geoffrey Chang	2013-07-31	[62]
4LSG	*M. musculus*	Hg^{2+}	None	3.80	0.357	Geoffrey Chang	2013-07-31	[62]
4M1M	*M. musculus*	Hg^{2+}	None	3.80	0.266	Stephen G. Aller	2013-11-13	[58]
4M2S	*M. musculus*	QZ59-RRR	None	4.40	0.294	Stephen G. Aller	2013-11-13	[58]
4M2T	*M. musculus*	QZ59-SSS	None	4.35	0.283	Stephen G. Aller	2013-11-13	[58]
3WMG	*C. merolae*[d]	Model ligands[e]	None	2.4	0.246	Hiroaki Kato	2014-04-30	[63]
3WME	*C. merolae*	DDM[f]	None	2.75	0.275	Hiroaki Kato	2014-03-19	[63]
3WMF	*C. merolae*	DDM	None	2.6	0.246	Hiroaki Kato	2014-03-19	[63]
3WMG	*C. merolae*	Model ligands[e]	None	2.4	0.246	Hiroaki Kato	2014-04-30	[63]
3WME	*C. merolae*	DDM	None	2.75	0.275	Hiroaki Kato	2014-03-19	[63]
3WMF	*C. merolae*	DDM	None	2.6	0.246	Hiroaki Kato	2014-03-19	[63]
3WMG	*C. merolae*	Model ligands[e]	None	2.4	0.246	Hiroaki Kato	2014-04-30	[63]
3WME	*C. merolae*	DDM	None	2.75	0.275	Hiroaki Kato	2014-03-19	[63]
3WMF	*C. merolae*	DDM	None	2.6	0.246	Hiroaki Kato	2014-03-19	[63]
4Q9H[g]	*M. musculus*	None	None	3.40	0.291	Geoffrey Chang	2015-03-04	[64]
4Q9I[g]	*M. musculus*	QZ-Ala	None	3.78	0.295	Geoffrey Chang	2015-03-04	[64]
4Q9J[g]	*M. musculus*	QZ-Val	None	3.60	0.282	Geoffrey Chang	2015-03-04	[64]
4Q9K[g]	*M. musculus*	QZ-Leu	None	3.80	0.321	Geoffrey Chang	2015-03-04	[64]
4Q9L[g]	*M. musculus*	QZ-Phe	None	3.80	0.293	Geoffrey Chang	2015-03-04	[64]
4XWK	*M. musculus*	DBE-100[k]	None	3.50	0.282	Geoffrey Chang	2015-01-29	[65]

a – Mus musculus.
b - Caenorhabditis elegans.
c - 2-(Acetylamino)-2-Deoxy-A-D-Glucopyranosel; N-Acetyl-D-Glucosamine; Beta-D-Mannose; Alpha-D-Mannose; Undecyl 4-O-Alpha-D-Glucopyranosyl-1-Thio-Beta-D- Glucopyranoside.
d- Cyanidioschyzon merolae.
e **-** Decyl-beta-D-Maltopyranoside; 2-Amino-2-Hydroxymethyl-Propane-1,3-Diol; anti-CmABCB1 peptide.
f **-** Decyl-beta-D-Maltopyranoside.
g - fully methylated protein.
h – Linker shortened by 34 residues.
i – methylated in the presence of AMP-PNP.
k - PBDE (polybrominated diphenyl ether)–100.

changes characterized by helix repacking during the ATP hydrolysis cycle [54, 55]. More recently, conformational landscape of mouse P-gp in solution and that for human P-gp in complex with a specific monoclonal antibody UIC2 was also explored by cryo-EM [56, 57]. Structural models of *h*P-gp in the open-inward conformations have been derived based on the full-length crystal structures of the mouse P-gp (*m*P-gp) [58], *C. elegance* P-gp (*ce*P-gp) [31], and that in the open–outward conformations from the bacterial ABC transporters (Sav1866 and MsbA) [59, 60]. Current structural information, especially for the open-outward conformation, is considered insufficient to provide necessary guidance for further mechanistic analyses of *h*P-gp in the realm of substrate and modulator interactions, due to low sequence identities and model defects. Successful structure determination of *h*P-gp has been considered a major goal in the ABC

transporter field in part due to its significance in cancer treatment, in the formation of many physiological barriers, and also due to difficulties in producing high quality protein preparations amenable to crystallization and in obtaining diffraction quality crystals.

In terms of mechanisms of ATP hydrolysis and its coupling to drug transport, it is likely that both human and mouse P-gp use highly homologous if not identical processes, given the 87% sequence identity between the two. Thus mouse P-gp can be used as a good model for understanding the function of human P-gp. Whether the mouse P-gp structure can be used to model drug interactions in human remains to be seen. So far there are 25 entries in the PDB databank for P-gp (Table **1**). Fifteen are for the structures of mouse P-gp, one for the nematode *C. elegans*, and nine for the red alga *C. merolae.*

CRYSTAL STRUCTURES OF MOUSE P-GP

In 2009, the first *m*P-gp structure (PDB:3G5U) was reported at 3.8 Å resolution determined by the MIR/AS (multiple isomorphous replacement with anomalous scattering) method and contained errors that were later corrected (PDB:4M1M) by the same authors after the structure of P-gp from *C. elegance* (*Ce*P-gp, PDB:4F4C) was reported (Figure **1**) [31, 58, 61]. The first report also contained structures of *m*P-gp in complex with cyclicpeptidal inhibitors QZ59-RRR (PDB:3G60) and QZ59-SSS (PDB:3G61) at lower resolution of 4.4 Å, which were also re-refined and re-reported (PDB:4M2S and 4M2T) [58]. One common feature for these structures was that they all showed an open-inward conformation with a distance of separation of 38.4 Å between the two NBDs, as measured between residue C427 of NBD1 and C1070 of NBD2. Clearly, *m*P-gp bears a strong topological similarity to the bacterial efflux transporters Sav1866 and MsbA, which were published earlier [59, 66], although these bacterial transporters are half transporters.

In addition to these structures there are three inward-facing conformations of *m*P-gp structures reported in 2013, which were derived from two different crystal forms [62]. The A-form was again determined by MAD phasing at 4 Å resolution (PDB: 4KSB & 4KSC). One structure at 4.1 Å resolution (PDB:4KSD) has a nanobody bound to the C-terminal side of the first NBD. This nanobody strongly inhibits the ATP hydrolysis activity of *m*P-gp by hindering the formation of a dimeric complex between the NBDs, which is essential for nucleotide hydrolysis. In a more recent publication,

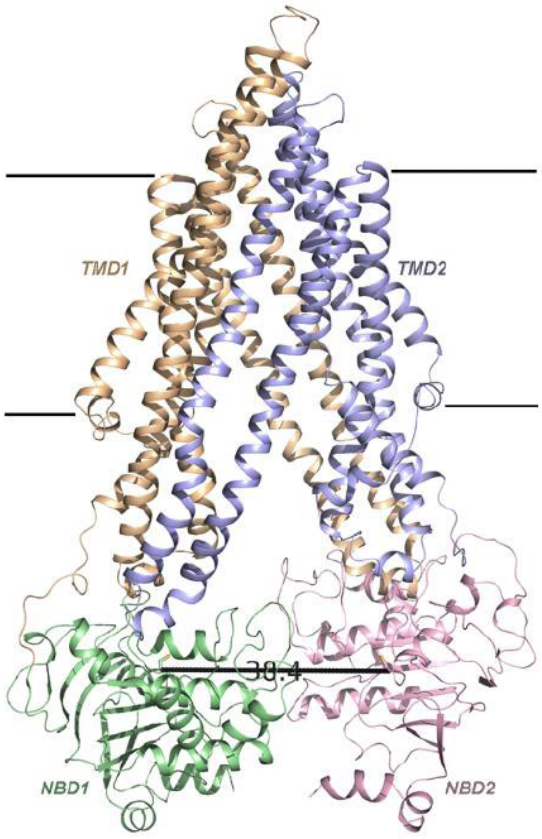

Figure 1: The ribbon representation of the structure of *m*P-gp (PDB:4M1M) in an inward-facing conformation. Major domains are shown in different colors and labeled. The two parallel black lines delineate the boundary of membrane bilayer. The separation between the two NBDs is measured by distance between the two conserved Walker A cysteine residues C427 and C1070.

methylated *m*P-gp structures were reported [64]. The methylated structure (PDB: 4Q9H) was determined at 3.4 Å resolution. Four co-crystal structures of methylated P-gp with a series of rationally designed ligands were also presented but at lower resolutions (PDB: 4Q9I, RQ9J, 4Q9K and 4Q9L). Interestingly, the binding of some, but not all, ligands produces a large conformational change in the TMH4.

CRYSTAL STURCTURE OF CLOSE HOMOLOGS OF P-GP

In addition to the *m*P-gp structures, two structures of P-gp homologs were reported. One is the structure of P-gp from the nemotade *Caenorhabditis elegans* (*Ce*P-gp), which was determined by SAD (single anomalous dispersion) phasing method at a resolution of 3.4 Å (Figure **2**) [31]. The transporter in the crystal structure features a very large separation (54 Å from C455 of NBD1 to C1116 of NBD2) between the two NBDs and opens its drug pathway at the level of the membrane's inner leaflet, presumably allowing drug to

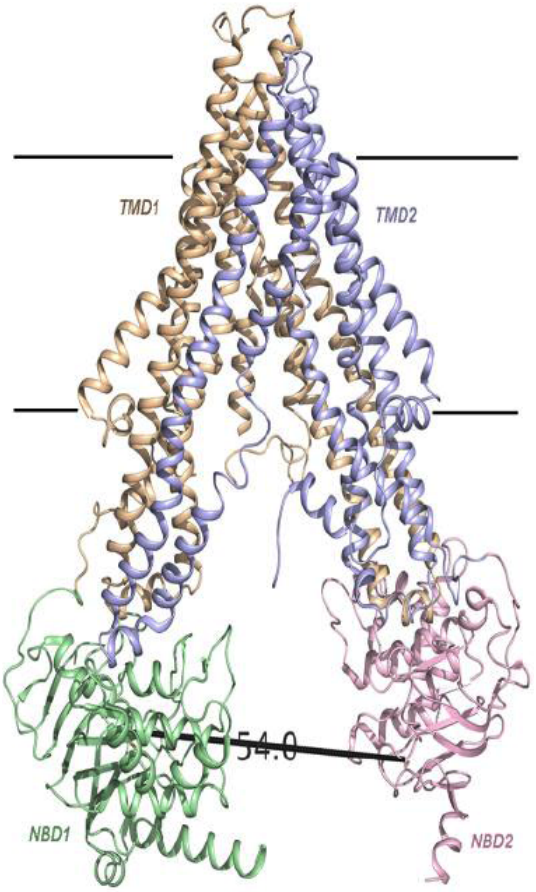

Figure 2: Cartoon representation of *Ce*P-gp structure. Structure of CeP-gp also revealed an inward-facing conformation with the two NBD domains separated by 54 Å, measured from two conserved cysteine residues C455 of NBC1 and C1116 of NBD2.

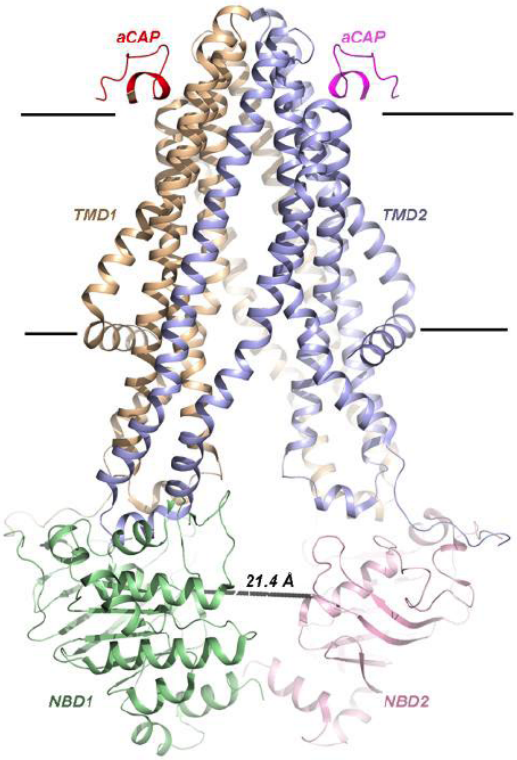

Figure 3: Ribbon representation of the structure of *CmABCB1*. This is a half transporter; thus it possesses a perfect 2-fold symmetry. The structure shown here has a bound peptide inhibitor aCAP on extracellular side of the protein. The structure was also determined in an open-inward conformation with the two NBDs separated by 21. 4 Å, as measured from two equivalent A482 residues that corresponds to C427 in *m*P-gp.

gain direct access from the membrane. In this structure, a ball-and-joint model was proposed for the interface between the TMDs and NBDs, similar to the ATP-binding cassette importers. Importantly, this structure led to a *h*P-gp model that suggested errors in the previously published *m*P-gp structure and led to the re-refinement of the first *m*P-gp [58].

A series of structures for a second P-gp homolog were reported for a primitive eukaryotic organism red alga *Cyanidioschyzon merolae* (*Cm*ABCB1, Figure **3**) [63]. Unlike typical eukaryotic P-gp, *Cm*ABCB1 is a half transporter and it lacks the characteristic cysteine residue in the Walker A motif of the NBD. The structures were determined using MAD (multiple anomalous dispersion) phasing method in two forms: an apo-form at 2.6 Å resolution and an allosteric inhibitor-bound form at 2.4 Å resolution (Figure **3**), both have the open inward conformation with the two NBDs separated by 21.4 Å, measured from A482 of one NBD to the equivalent residue of the other NBD. The residue A482 corresponds to C427 of *m*P-gp. The bound inhibitor clamps on the TM helices from the extracellular side, thus was believed to fix the *Cm*ABCB1 structure in an open-inward conformation, although this conformation is very similar to that of the unbound structure. These structures reveal the detailed architecture of the transporter, including a possible gate that opens to extracellular side and two gates that open to intramembranous region and the cytosolic side.

STRUCTURES OF P-GP WITH BOUND SUBSTRATE AND INHIBITORS

So far, there is no reported P-gp structure that has a nucleotide bound, which perhaps has something to do with low affinity binding of nucleotide to P-gp. Reported apparent Km values for ATP binding are in the range of 0.2 to 0.5 mM [44, 67]. Structures of *m*P-gp in complex with cyclic-peptide inhibitors (or QZ compounds) were reported for wild-type and methylated *m*P-gp [58, 64]. Multiple binding sites and different binding modes for the same QZ compound have been observed. More recently, the structure of methylated *m*P-gp in complex

with an environmental pollutant, PBDE (polybrominated diphenyl ether)–100, was published (Figure **4**) [65]. Interestingly, in all the structures of *m*P-gp/inhibitor complexes, inhibitors bind to the same general locations near the tip of the wedge formed by the two halves of the molecule (Figure **4**), which were also observed by molecular docking experiments with a number of P-gp substrate/inhibitors [68]. The binding sites so far obtained are aligned with the outer leaflet of the cytoplasmic membrane; no substrate-binding site has been shown to align with the inner leaflet of the membrane.

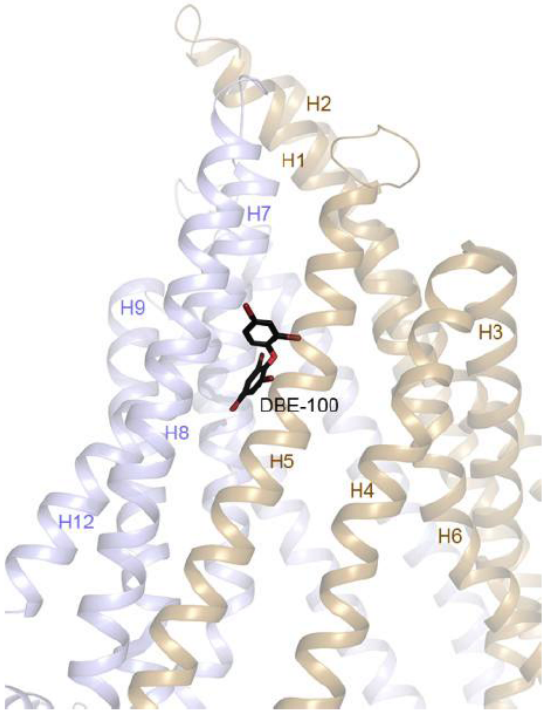

Figure 4: Binding of PBED-100 to methylated *m*P-gp. Only the TM domains are shown in cartoon rendition with TMD1 in orange and TMD2 in purple. TM helices are labeled. The bound inhibitor is shown as stick model with carbon in black and oxygen in red; it is bound at the interface between helices H5 and H8.

STRUCTURAL STUDIES OF P-GP BY EM

Cryo-electron microscopy (Cryo-EM) has emerged as a credible contender to experimentally achieve atomic resolution information for large biological molecules and complexes. In principle, cryo-EM can quickly create high-resolution models of molecules that have resisted X-ray crystallography and other approaches and there is a range of very important biological problems that are now possibly open to being tackled in a way that they could never before. Recent examples of the application of cryo-EM on ABC transporters are the structure determination of bacterial exporter TmrAB from the thermophilic Gram-negative eubacterium *Thermus thermophiles* at 8.2 Å resolution and that of TAP hetero dimer, transporter associated with antigen processing, in complex with a viral inhibitor ICP47 determined at 6.5 Å resolution [69, 70].

A couple of recent cryo-EM studies on both *m*P-gp and *h*P-gp were reported; both were at relatively low resolutions. In one report, single particle EM was used to directly visualize the conformational spectrum of *m*P-gp that was stabilized by amphiphiles in a lipid bilayer-mimicking environment. In this work, EM imaging and analysis using an unbiased approach to 3D model construction was used to delineate the entire conformational spectrum of P-gp and the influence of nucleotide and substrate binding. This analysis revealed differences between the bacterial and mammalian transporters regarding the effect of binding nucleotides and substrates on their conformational changes and the range of NBD separations across the entire structural spectrum [56]. In another study, cryo-EM was used to evaluate the conformations of *h*P-gp bound with a Fab fragment of the specific monoclonal antibody UIC2 in four nucleotide-binding states of its ATPase cycle. Despite low resolution, *h*P-gp in its apo state can coexist in two conformations, where the NBDs are either separated (inward-facing conformation) or in close proximity (probable outward-facing conformation). These coexisting conformations are present even in the presence of bound ATP prior to hydrolysis. Interestingly, the transporter trapped in the ADP state by vanadate showed exclusively open-outward conformation [57].

Despite the best attempts by highly reputable laboratories, structures of ABC transporters, especially those of P-gp, obtained by cryo-EM method remain at relatively low resolution, which was also manifested in the structural studies of P-gp by X-ray crystallography and most likely caused by the intrinsic flexibility of the molecules in detergent solutions. Furthermore, this problem is further aggravated for cryo-EM by the intrinsic pseudo two-fold symmetry of P-gp that so far can only be circumvented by tagging the protein asymmetrically. Future structure determination of P-gp by cryo-EM requires development of methods that can effectively fix the conformation flexibility of P-gp.

INSIGHT INTO THE MECHANISM OF P-GP FUNCTION REVEALED BY THE AVAILABLE STRUCTURES

Clearly, available P-gp structures are consistent with the general mechanism of the alternating access

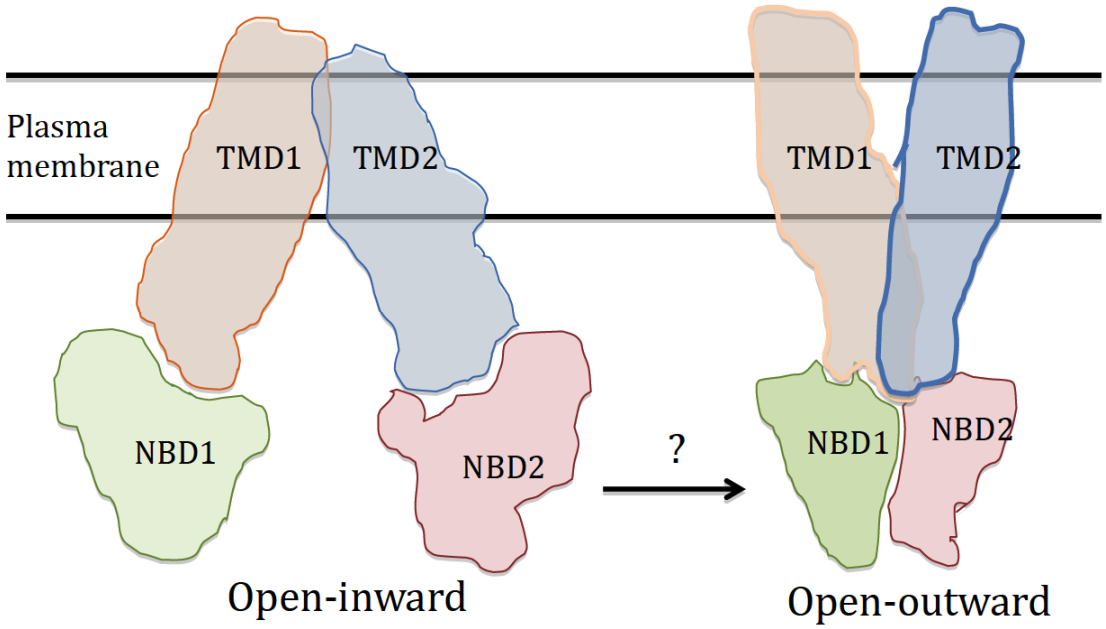

Figure 5: Mechanistic insight of P-gp function based on structural analysis of P-gp. Two different conformations of P-gp are illustrated: Open-inward and Open-outward. While the open-inward conformations were observed rather frequently, that of open-outward has so far not been observed experimentally.

model put forward decades ago [71]. However, the fact that most structures determined to date, by both crystallography and cryo-EM, display an inward-facing conformation seems to suggest that P-gp in detergent solution are more stable with the open-inward conformation, which is rather different from bacterial ABC transporters under similar conditions showing nucleotide dependent conformation switch. In other words, the presence of nucleotide or ATP alone is not sufficient to drive apo P-gp to the open-outward conformation in detergent solution. It is also interesting to notice that in the absence of substrate, P-gp takes open-outward conformation under post-ATP hydrolysis conditions [57]. It is conceivable that P-gp may assume a stable open-outward conformation when substrates bind to the protein in membrane.

FUTURE PERSPECTIVE

For the past few years we have seen significant progress made for the structure determinations of P-gp and its close homologs. For the coming years, we have every reason to expect that more P-gp structures will appear at higher resolution and with better quality. Structures of P-gp with bound nucleotides and with various bound compounds will also come to light. The processes of structure determinations as reported in the literature revealed that most structures were determined by experimental phasing methods. In other words, despite high similarity in sequence, these structures are dissimilar enough that experimental phasing methods had to be employed in the course of structure determination, which defies the common practice in protein crystallography and begs for an answer of their functional significance. Moreover, the questions we should be asking now are whether these structures represent the majority or only a small portion of the conformational landscape of P-gp that is necessary for its drug transport function and how these structures can be used to provide mechanistic insights into the function of P-gp. There will be an exciting time ahead for harvesting important functional significance from these structures. Most importantly, we ought to be thinking about how structural information can be used to control the function of P-gp for the benefit of human life and health.

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