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Substrate polyspecificity and conformational relevance in ABC transporters: new insights from structural studies

Jack Wright[1], Stephen P. Muench[1], Adrian Goldman[1,3], Alison Baker[1,2*]

[1]Astbury Centre for Structural Molecular Biology, School of Biomedical Science, University of Leeds, LS2 9JT, U.K

[2]Centre for Plant Sciences, School of Molecular and Cellular Biology, University of Leeds, LS2 9JT, U.K

[3]Division of Biochemistry, Department of Biosciences, University of Helsinki, Helsinki, FIN-00014, Finland

[*]**To whom correspondence should be addressed (email a.baker@leeds.ac.uk)**

Abbreviations: ABC, ATP-binding cassette; ATP, adenosine triphosphate; CFTR, cystic fibrosis conductance regulator; cryo-EM, cryo-electron microscopy; CTS, comatose; DDM, N-dodecyl-β-D-maltoside; EPR, electron paramagnetic resonance; LMNG, lauryl maltose neopentyl glycol; LPS, lipopolysaccharide; LTC4, leukotriene C4; MDR, multi-drug resistance; MRP1, multi-drug resistance protein 1; NBDs, nucleotide-binding domains; P-gp, P-glycoprotein; TMDs, transmembrane domains; X-ALD, X-linked adrenoleukodystrophy

Abstract

Transport of molecules and ions across biological membranes is an essential process in all organisms. It is carried out by a range of evolutionarily conserved primary and secondary transporters. A significant portion of the primary transporters belong to the ATP-binding cassette (ABC) superfamily, which utilise the free-energy from ATP hydrolysis to shuttle many different substrates across various biological membranes, and consequently, are involved in both normal and abnormal physiology. In humans, ABC-transporter associated pathologies are perhaps best exemplified by multidrug-resistance transporters that efflux many xenobiotic compounds due to their remarkable substrate polyspecificity. Accordingly, understanding the transport mechanism(s) is of great significance, and indeed, much progress has been made in recent years, particularly from structural studies on ABC exporters. Consequently, the general mechanism of 'alternate access' has been modified to describe individual transporter nuances, though some aspects of the transport process remain unclear. Moreover, as new information has emerged, the physiological relevance of the 'open-apo' conformation of MsbA (a bacterial exporter) has been questioned, and by extension, its contribution to mechanistic models. We present here a comprehensive overview of the most recently solved structures of ABC exporters, focusing on new insights regarding the nature of substrate polyspecificity, and the physiological relevance of the 'open-apo' conformation. This review evaluates the claim that the latter may be an artefact of detergent solubilisation, and we hypothesise that the biophysical properties of the membrane play a key role in the function of ABC exporters allowing them to behave like a 'spring-hinge' during their transport cycle.

Introduction

ATP-binding cassette (ABC) transporters constitute a superfamily of membrane proteins that couple the hydrolysis of ATP to vectorial transport of substrates across biological membranes [1]. Transporters, which can be importers or exporters (Fig. 1), transport a wide variety of substrates ranging from maltose by MalFGK2 [2] to entire macromolecules such as Lipopolysaccharides (LPS) by MsbA [3], oligosaccharides by PglK [4,5], and type 1 secretion system (T1SS) substrate proteins by HlyB [6]. Individual transporters can be highly specific or highly promiscuous; for example, importers such as BhuT, a bacterial haem transporter, typically associate with a specific substrate binding protein to initiate transport (Fig. 1, right [7]) whereas many exporters such as Sav1866, a bacterial exporter,(Fig. 1, left [8]) show a high degree of substrate polyspecificity. Importantly, the ability of many ABC exporters to transport a variety of xenobiotic substrates means that they contribute significantly to multidrug resistance (MDR) in both prokaryotes and eukaryotes [9]. Moreover, the ubiquity of ABC transporters means that they are implicated in many clinically significant pathologies including cancer, cystic fibrosis (CF), and, in the case of family D transporters, X-linked adrenoleukodystrophy (X-ALD [10–13]). That ABC transporters are not confined exclusively to the plasma membranes [14–16] certainly contributes to their clinical importance.

ABC transporters are characterised by a common domain structure consisting of two transmembrane domains (TMDs), each consisting of multiple transmembrane α-helices, followed by two nucleotide-binding domains (NBDs) that extend into the cytosol (Fig. 1). The TMDs and NBDs are linked by a 'coupling helix' that sits in a crevice on the NBD surface: this motif permits coupling of nucleotide hydrolysis to substrate transport by channelling the conformational change through the transporter. In all cases, functional transporters result from dimerization of the NBDs, where the nucleotide binding sites (NBSs) themselves are composite domains created by the interactions of key conserved motifs from each half of the transporter [17]. Once ATP is sandwiched between the NBDs, the transporter adopts a closed inward facing conformation. According to the alternate access model,

nucleotide and substrate binding converts the transporter to an outward-occluded state followed by rearrangement of the core helices that expel substrate to the extracellular space. Subsequently, ATP hydrolysis and release of inorganic phosphate converts the transporter back to an inward facing conformation, this time with NBDs pushed apart ready for another cycle. A considerable body of research supports the 'alternate access' model of ABC exporters where the transporter interconverts between inward-facing and outward-facing conformations [9,18,19] during the transport cycle. However, it has become clear from more recent biochemical and, in particular, structural data that transporter-specific features/observations eliminate the possibility of a one-size-fits-all model to describe the mechanism of all transporters. Whilst useful in describing a general transporter mechanism, the simplicity of alternate access has led to a number of other models being proposed, including the switch model [20] , hydrophobic vacuum [21] , outward-only [4,5], and constant-contact [22]. Whilst this review will focus on transported as oppose to catalytic substrates, readers interested in the role of nucleotide during transport, are directed to the following articles [4,20–24]. Moreover, the physiological relevance of some early MsbA crystal structures (and indeed one structure of PglK) – specifically those with widely separated NBDs [4,25] – has been questioned in recent years, despite their role in developing mechanistic models of ABC exporters [1]. While structural data have been of vital importance in addressing these observations, a number of outstanding questions have remained. Two key questions are: how does a single transporter bind so many different substrates? How physiologically relevant are the wide open inward-facing conformations? Accordingly, the aim of this review is to shed light on these questions by discussing new insights from recently solved structures of ABC exporters.

Substrate Binding

The role of ATP as a catalytic substrate for ABC transporter has been well documented [1,17,23,24,26,27] , therefore the focus of this review will be the transported substrates (from here referred to simply as 'substrates'). Despite the success in identifying the substrates of ABC transporters, the lack of bound substrates in the available structures has limited our understanding of the molecular basis of substrate promiscuity. The recent cryo-EM structures of bovine MRP1 – apo and with bound leukotriene C4 (LTC4) [28] – have revealed important insights into the mode of substrate binding and polyspecificity, and are in remarkably good agreement with earlier biochemical studies [29]. Binding of LTC4, a physiological substrate of MRP1, occurs through a bipartite cavity divided into hydrophobic (H-pocket) and hydrophilic regions (P-pocket), where the LTC4 lipid tail is accommodated by the former and the glutathione moiety by the latter. The fact that the P-pocket is made up of residues from both halves of the transporter, whereas the H-pocket residues come only from TM bundle 2 indicates how different substrates might bind MRP1 (Fig. 2) *i.e.* amphipathic substrates can bridge the two halves of the transporter to align the NBDs for ATP hydrolysis. The rearrangement of MRP1 TMDs residues to interact with LTC4 (observed between LTC4 and apo structures) may indicate MRP1 plasticity and explain its ability to transport multiple substrates. This kind of TMD rearrangement has been implied from hydrogen-deuterium exchange experiments and comparison of a variety of crystal structures from another MDR transporter, P-gp [30,31]. By rearranging the TMDs through binding, twisting or unwinding, MDR ABC transporters may be able to tune the surface topology of the substrate binding cavity, thus providing a basis for substrate polyspecificity.

Like MRP1, MsbA has been shown to bind its substrate via a bipartite cavity. Two nucleotide-free structures of MsbA have been determined in the last two years: one with the protein inserted in nanodiscs and solved by cryo-EM [32], and another using facial amphiphiles and solved by x-ray crystallography [33]. In both cases, these structures revealed LPS bridged between the two halves of the transporter (like LTC4 in MRP1, above) via a hydrophobic pocket and a hydrophilic cavity, divided

by a ring of residues interacting with the glucosamines of LPS. The higher resolution of the crystal structure compared to the cryo-EM structure (2.9 Å vs. 4.7 Å, respectively) significantly improved the placement of the entire LPS molecule. This highlighted the ability of the transporter to accommodate lipid A but not bulk membrane phospholipids, thus explaining substrate selectivity within the MsbA cavity. Earlier work [34] proposed that in addition to lipid A, MsbA can bind amphipathic compounds at a second binding site and that this may cause an alteration in protein conformation. Moreover, fluorescence-quenching [35] and solid-state NMR experiments [36] have implied that this binding site resides within the TMDs, and not the central cavity of the transporter (*i.e.* the putative LPS binding site). This hypothesis is supported the recent MsbA crystal structure [33], where the inhibitors, G907 and G092, were bound to membrane-exposed crevices in both monomers of the transporter with G907-induced uncoupling of the NBDs from the TMDs.

Although ABCG2 is from a different family to MRP1 and MsbA, two recently solved cryo-EM structures (one reconstituted into a nanodisc and bound to cholesterol, and another reconstituted into a nanodisc and bound to inhibitor [37,38]) have revealed that the substrate binding site is divided into two regions: cavity one is open to the cytoplasm/inner-leaflet of the bilayer and is distinctly hydrophobic, whereas cavity two sits above the first and is relatively less hydrophobic. The cavities are separated by a 'leucine-plug' comprising L554 from opposing monomers. In the first structure of ABCG2 (representing the first structure of a family G ABC transporter [37]), the authors assign ambiguous density within cavity 1 to bound cholesterol (present during reconstitution into nanodiscs) that, while not an archetypal ABCG2 substrate, is indeed an amphipathic molecule. In the second, higher resolution structure [38], density for an inhibitor was observed at the exact position where cholesterol was placed in the previous structure, supporting the ability of this cavity to bind flat, polycyclic, amphipathic molecules. Together these data support a model in which substrate binding to cavity 1 induces dimerisation of the NBDs, followed by ATP binding and hydrolysis. Hydrolysis induces conformational changes in the TMDs via the coupling helix that causes rearrangement of the leucine-plug, allowing substrate to enter into cavity 2, where a hydrophobic mismatch results in expulsion of

the substrate into the extracellular space. This model may in part explain substrate promiscuity of ABCG2, [39], though the wide variety of substrates including steroid conjugates, tyrosine kinase inhibitors, and topoisomerase inhibitors, many of which are amphipathic but not all of which are flat polycyclic molecules leaves questions unanswered. The accompanying article by Kapoor *et al*, discusses substrate binding by ABCG2 in more detail and proposes a hypothetical model for multidrug efflux by this important protein [Kapoor].

Physiological Relevance of Widely Separated NBDs

The physiological relevance of inward-facing conformations, specifically those with widely separated NBDs, is frequently disputed due to inconclusive evidence on both sides of the debate as discussed by Locher [1]. A number of factors could potentially lead to artefactual results including absence of nucleotide, lack of transport substrate, detergent solubilisation, and crystallisation conditions meaning caution must be exercised in interpretations. However, the range of new structures has provided some interesting insights into this key issue. While four MsbA crystal structures were available before 2017 [25], none of them contained substrate. These structures, which were all solved after solubilising the transporter using maltoside detergents, adopted three specific conformations: 'nucleotide-bound' (in an outward-facing state), 'closed-apo' (an inward facing conformation with NBDs in close proximity), and open-apo' (with widely separated NBDs).

Both the cryo-EM and crystal structures of MsbA reported in 2017/18 (above, [32,33]) are nucleotide-free but LPS-bound, and adopt an inward facing conformation most similar to the 'closed-apo' conformation observed by Ward *et al.* [25] (Fig. 3). To date, no other structure of MsbA has been solved in the 'open-apo' conformation. Moreover, nucleotide-free nanodisc-reconstituted ABCG2 was also observed in an inward-facing conformation when bound by cholesterol or inhibitor[37,38]. Mi *et al.* [32] described another nanodisc-reconstituted MsbA structure with bound ADP-vanadate which they interpret as a novel outward-occluded form of MsbA (Fig. 3). This is in contrast to the ADP-vanadate crystal structure solved by Ward *et al.* [25] in which the transporter adopts a typical outward-facing conformation (this difference may be explained by substrate binding in the nanodisc structure). The 'apo-inward' crystal structures of detergent-solubilised PglK[4] are most similar to the 'open-apo' crystal structure of detergent-solubilised MsbA, with widely separated NBDs (Fig. 3).

To reconcile these differences (and explain why one may observe the 'open-apo' conformation vs. a typical inward-facing state with NBDs in close proximity), one must consider the solubilisation method used and the fact that some transporters are substrate or nucleotide bound. In both the cryo-EM and

crystal structures of MsbA solved in 2017/18 and in the cryo-EM structure of ABCG2, the two halves of the transporters are bridged by the substrates, LPS and cholesterol, respectively. This limits the angle through which the two NBDs can separate; thus, on the basis of substrate binding alone, it is not possible to discount the physiological relevance of the 'open-apo' crystal structure[1,25]. Moreover, the 'open-apo' conformation of the MsbA crystal structure was predicted from electron paramagnetic resonance (EPR) experiments in detergent[40,41] and liposomes[42].

However, preliminary negative stain data of MsbA solubilised in DDM by Mi *et al.* [32] exhibited features similar to the 'apo-open' crystal structure, as did low-resolution electron microscopy of ABCG2 solubilised using DDM [43], indicating that detergent solubilisation might favour such conformations. This may explain the EPR data in detergent (above), since this was carried out on DDM-solubilised MsbA, but does not explain the observation of the 'open-apo' conformation of MsbA in liposomes [42]. Care must thus be taken when considering this negative stain data given the limited resolution and possible staining artefacts, *e.g.* structure flattening and deformation. However, as the 'open-apo' conformation was not observed in any of the 2D classes used to solve the cryo-EM MsbA structure [32], nor indeed in the cryo-EM structures of ABCG2 [37,38], both of which were solved in nanodiscs, the 'open-apo' conformation may be an artefact of detergent solubilisation. This is not unlikely given that the 'open-apo' conformation has never been observed in the 3D structure of any transporters when either bound to substrate and/or when reconstituted into nanodiscs. Although one may argue that nanodiscs may artificially constrain the extent to which the transporter can open, Mi *et al* reported evidence to the contrary *(i.e.* increasing the size of the nanodisc did not alter conformation of MsbA observed in both negative-stain and cryo-EM).

Reconstituted proteins, whether in nanodiscs or liposomes may be in a more native-like environment due to the presence of lipids and subsequently a conformation that better reflects the native state. However, due to the initial detergent solubilsation step only very tightly bound native lipids might be retained. Detergent-free solubilisation methods such as SMALPs hold much promise in addressing the

role of lipids [44] since, unlike nanodiscs, there is no need to artificially add lipids back to the protein. Given that detergent poorly mimics the physiological environment, it is likely that the cryo-EM structures of MsbA solved in nanodiscs provide a more accurate description of MsbA than do the previous crystal structures [25]. This hypothesis is likely to extend to nanodisc-reconstituted crystal structures of PglK, ABCG2, P-glycoproteinr [44,45] since it is generally accepted that nanodiscs provide a more native environment than do detergents [45–47].

The properties of the lipid bilayer have been shown to affect membrane protein function [48]. The bilayer deformation energy contributes to the free energy associated with protein conformational changes. So, alterations to the bilayer or lipid environment surrounding the membrane protein that alters the bilayer deformation energy may modulate the conformation of the membrane protein [49]. Indeed, this may result from changes in membrane lateral pressure, as well as bilayer thickness, elasticity, and intrinsic curvature [50,51], discussion of which is beyond the scope of this review (but is available in the following article [48]). Nonetheless, one hypothesis to explain the observed difference between detergent-solubilised and nanodisc-reconstituted structures is that the aforementioned membrane properties are markedly different between lipid bilayers and detergent micelles, and consequently each will favour a particular conformation of the transporter; in the case of a micelle, the transporter is able to behave like a spring-hinge, popping open and allowing the NBDs greater separation (‘open-apo’). Additionally, the high curvature of the micelle might allow penetration of water molecules into the micelle interior, weakening its overall structure and/or perhaps promoting non-native like interactions with the protein [52] (clearly, this is highly dependent on the detergent used, which perhaps explains the inconsistency in ATPase activity reported for detergent solubilised P-gp discussed below). Conversely, when reconstituted into a nanodisc, the transporter exists in a more stable environment surrounded by a membrane protein scaffold that may be capable of holding the NBDs closer together (Fig. 4). This may be synergistic with the formation of a bilayer during nanodisc reconstitution since bilayer thickness is known to place a crucial role in modulating membrane protein function (one cannot easily define thickness when considering a micelle due to its

intrinsic curvature). This hypothesis is supported by small angle scattering studies (neutron and x-ray) of nanodisc-reconstituted MsbA and by luminescence resonance energy transfer experiments of nanodisc-reconstituted P-gp treated with substrate, in which the wide 'open-apo' conformation could not be observed [53,54]. In addition, the structures of ABCG5/G8 [55], the peptidase-containing ATP-binding cassette transporter (PCAT1; [56]) and the maltose transporter–EIIA complex [2] all adopt an inward-facing conformation with NBDs in close proximity when crystallised in the presence of lipids (*i.e.* in bicelles). More specifically, ABCG5/G8 adopts this conformation in the absence of nucleotide and substrate, suggesting that the lipid favours a 'closed-apo' conformation (or that ABCG family transporters adopt this conformation at rest). Moreover, the 'open-apo' conformation was not observed in the crystal structure of MsbA solubilised using a facial amphiphile[57]. These structurally unique detergents are known to form smaller and tighter micelles than that of DDM, perhaps constricting the movement of TMDs and preventing wide separation. Finally, the 'open-apo' conformation was observed in two crystal structures of the lipid flippase, PglK, after solubilisation of the transporter using LMNG or DDM, but was not seen when the NBDs were locked together by binding of ADP-vanadate [4] or by a conformation-specific nanobody [5]. Hence, the report by Perez *et al.* [4] that PglK exhibits negligible ATPase activity when solubilised in detergent is likely due to the 'open-apo' conformation being the predominant form in solution after purification *i.e.* the substitution of the native lipid bilayer for the detergent micelle favours the 'open-apo' conformation and allows the transporter to behave as a spring-hinge. This may also be true of MsbA since both Mi *et al* [32] and Kawai *et al* [58] reported greater ATPase activity of MsbA when reconstituted into nanodiscs than in detergent. However, the reported ATPase activity of detergent solubilised P-gp has been inconsistent, with some groups observing inhibition, and others observing peak activity [59,60]. This conflict may be explained by the choice of detergent used for solubilisation, in this case DDM vs. CHAPS; due to difference in cross-sectional area between detergent head & tail groups, as well as the electrostatic repulsion between the former, one must consider the degree to which micelle curvature, and curvature frustration play a role in maintaining the transporter in an active state [52] as has been

noted for Rhodopsin [61]. Interestingly, by altering the lipid composition of a nanodisc, studies on the $MalFGK_2$ complex have implicated curvature and membrane thickness as a regulator of this transporters activity [62]. Thus, some transporters may be suitable for study using specific detergents, while others must first be reconstituted into a more membrane-like environment. This latter point may play a significant role in future studies as we develop our understanding of the role of the lipid environment surrounding ABC transporters and its role in structure/function.

Conclusion

The recently solved crystal and cryo-EM structures of ABC transporters have not only shed light on the mystery of substrate polyspecificity, but have crucially highlighted how sample handling could affect the experimental outcome. The hypothesis that the 'open-apo' conformation can be an artefact of detergent solubilisation may be disproved if the structure of an apo ABC exporter displaying widely separated NBDs is determined in a membrane-like environment. Given the speed with which the field is moving, many of these questions will no doubt be addressed as new structures become available. It also seems likely that not all ABC transporters are equally sensitive to the effect of detergent solubilisation as evidenced by differing rates of ATPase activity in detergent micelles. Nonetheless, it is clear that one should exercise caution when incorporating a newly solved structure into a new mechanistic model if the conformation is an inward one. Finally, it is clear from the direction of the field of structural biology as a whole that the role of the membrane and local lipid environment is crucial for optimal membrane protein function. Thus, the hypothesis that ABC transporters behave in a spring-like fashion and are regulated by biophysical properties of the membrane should be considered when devising new mechanistic models.

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Competing Interests

We declare that there are no competing interests associated with this review

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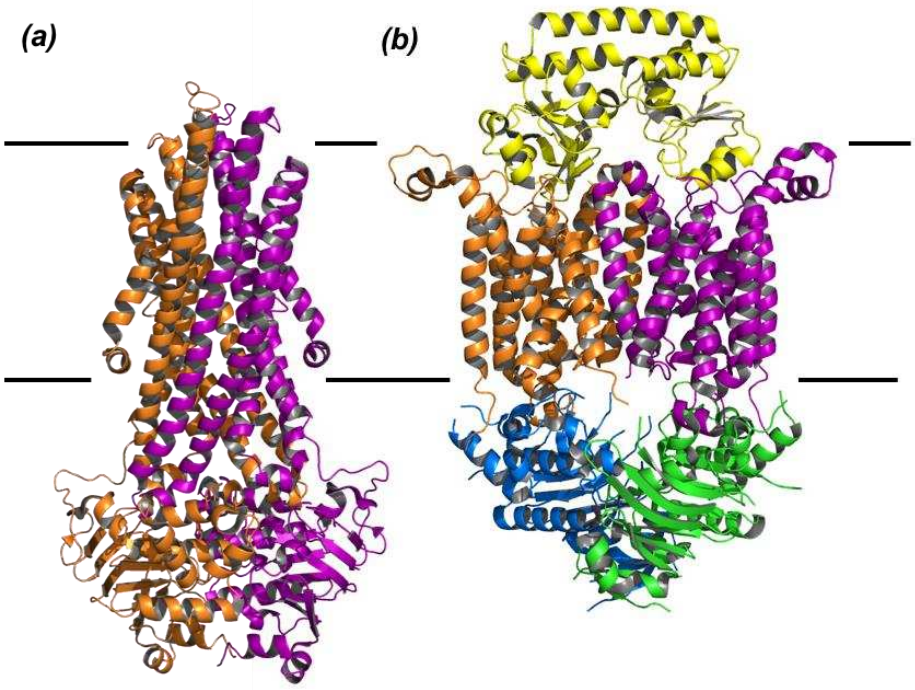

Figure 1. Example structures of ABC exporters and importers (a) Exporter, Sav1866 (PDB ID: 2HYD). (b) Importer, BhuT (PDB ID: 5B58). For Sav1886; monomers (each containing one TMD and one NBD) are shown in purple and orange. For BhuT; TMDs, purple and orange; NBDs, blue & green; substrate-binding protein, yellow. Black lines denote the membrane.

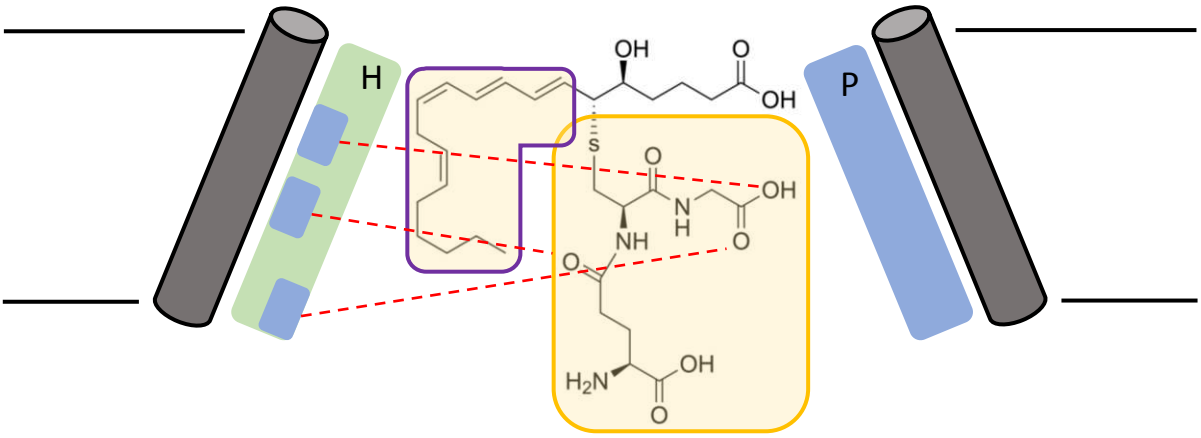

Figure 2. The substrate binding site of MRP1 showing the physiological substrate LTC4. Residues from the P-pocket (blue) are found in both halves of the transporter and interact with the glutathione moiety of LTC4 (orange outline), while residues from the H-pocket (green) are found only in one half of the transporter and interact with the lipid tail of LTC4. Helices of the transporter cavity are shown as black cylinders surrounded by the membrane (parallel black lines).

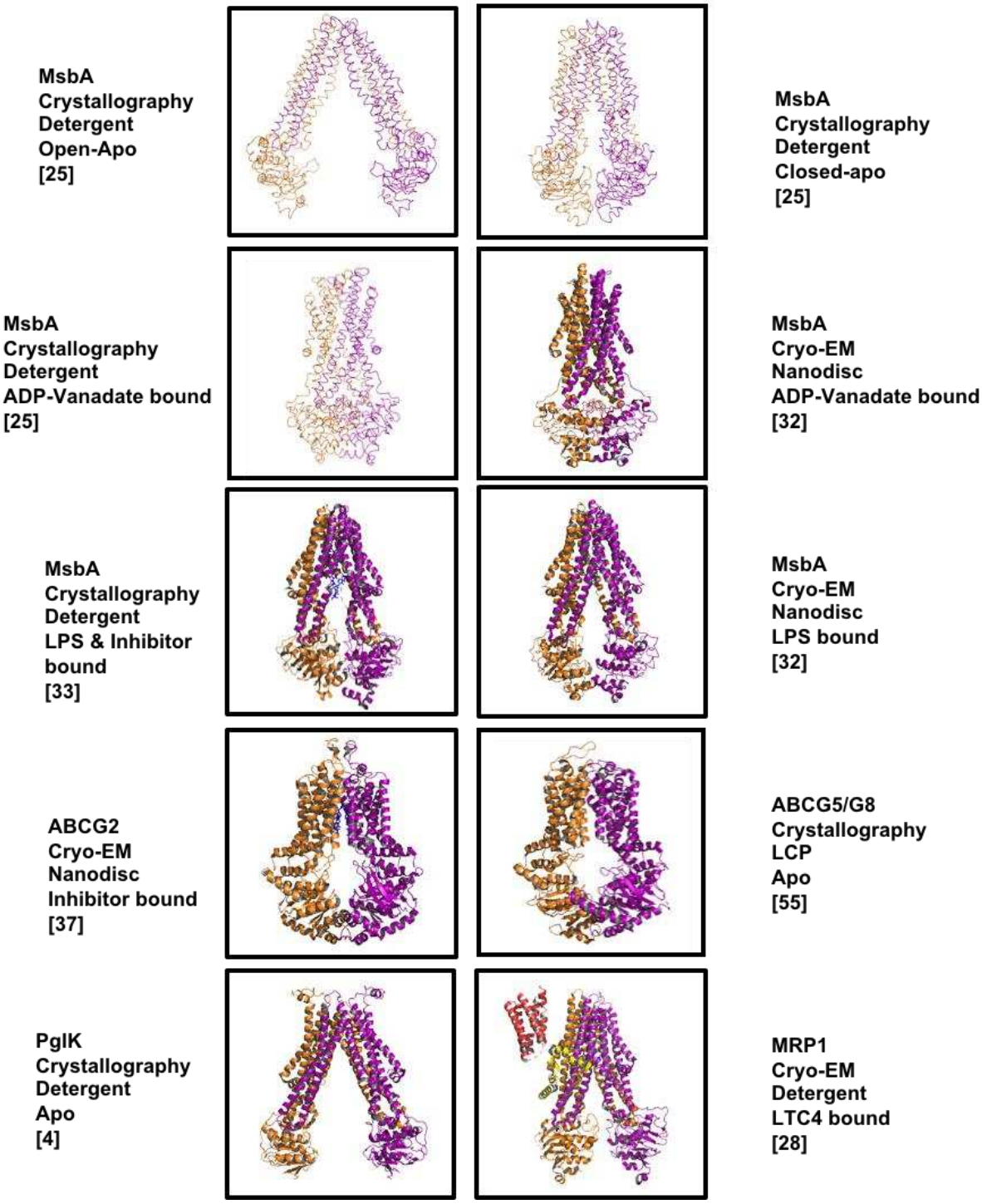

Figure 3. A comparison between the MsbA structures solved by Ward et al., 2007 - showing the 'open-apo' conformation - with more recently solved structures. Transporter monomers are shown in purple and orange. In the case of MRP1, which folds from a single polypeptide chain, the same colour scheme has been adopted for clarity. In addition, the TMD0 and the lasso motif of MRP1 are shown in red and yellow respectively. Ward et al [25] deposited only the C-alpha trace into the PBD, hence the different representation.

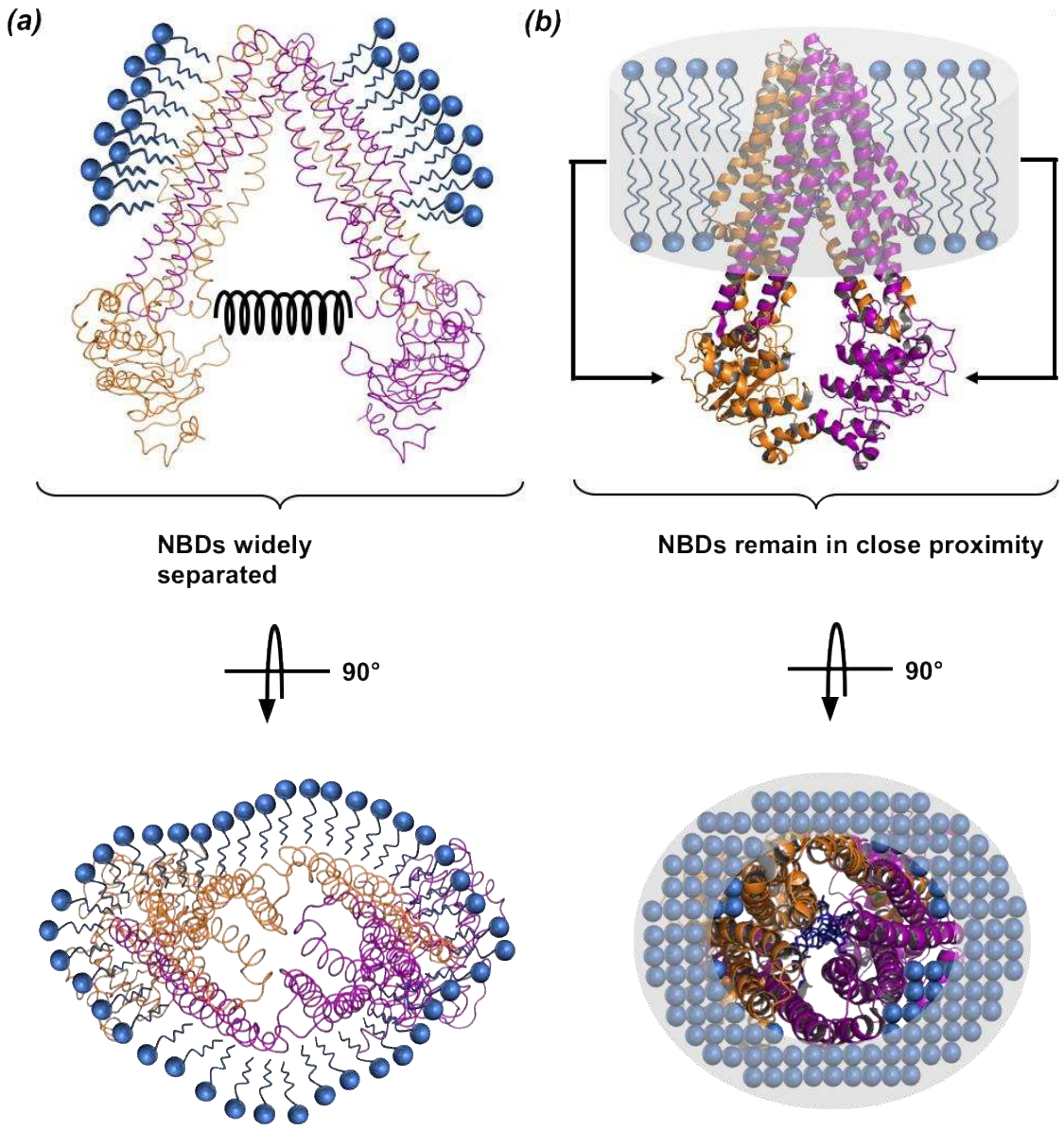

Figure 4. The 'open-apo' conformation of ABC exporters may be an artefact of detergent solubilisation. (a) Crystal structure of MsbA solved by Ward et al. (2007) solubilised in detergent (C_a trace only); detergent molecules strip away the native lipids surrounding the membrane protein and form a micelle around exposed hydrophobic regions. The physical properties of the detergent micelle favour a conformation in which the transporter NBDs are spaced widely apart, perhaps due to their behaving like a springe-hinge (black horizontal helix). (b) Cryo-EM structure of MsbA solved by Mi et al. (2017) when reconstituted into nanodiscs; compared to detergent, the more native environment of the nanodisc favours a compact transporter conformation, with NBDs in close proximity. Given that the activity of ABC transporters is generally enhanced when reconstituted into nanodiscs, this conformation is more likely to be physiologically relevant than the wide-open conformation Transporter monomers; purple and orange; detergent; blue, one tail; lipids, blue, two tails; nanodisc annulus, grey halo.