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REVIEW ARTICLE

What monomeric nucleotide binding domains can teach us about dimeric ABC proteins

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The classic conceptualization of ATP binding cassette (ABC) transporter function is an ATP-dependent conformational change coupled to transport of a substrate across a biological membrane via the transmembrane domains (TMDs). The binding of two ATP molecules within the transporter's two nucleotide binding domains (NBDs) induces their dimerization. Despite retaining the ability to bind nucleotides, isolated NBDs frequently fail to dimerize. ABC proteins without a TMD, for example ABCE and ABCF, have NBDs tethered via elaborate linkers, further supporting that NBD dimerization does not readily occur for isolated NBDs. Intriguingly, even in full-length transporters, the NBD-dimerized, outward-facing state is not as frequently observed as might be expected. This leads to questions regarding what drives NBD interaction and the role of the TMDs or linkers. Understanding the NBD–nucleotide interaction and the subsequent NBD dimerization is thus pivotal for understanding ABC transporter activity in general. Here, we hope to provide new insights into ABC protein function by discussing the perplexing issue of (missing) NBD dimerization in isolation and in the context of full-length ABC proteins.

Keywords: ATP binding cassette; dimerization; domain interactions; domain linkers; membrane transporter; nucleotide binding domain; protein structure and function

ATP binding cassette proteins

ATP binding cassette (ABC) proteins are a fascinating protein family present in all phyla of life. Members of the human ABC protein family divide into seven subfamilies (ABCA-G) with distinct cellular functions [1]. With the exception of the ABCE and ABCF subfamilies, which are soluble proteins consisting of two linked nucleotide binding domains (NBDs) [1], and the ABCC7, ABCC8 and ABCC9 proteins that are involved in channel function or regulation, all other human ABC proteins are thought to be membrane transporters. Their NBDs, which are structurally highly conserved and contain numerous motifs important for ATP binding, hydrolysis and interdomain communication, are tethered to transmembrane domains (TMDs) responsible for substrate interaction and translocation (Fig. 1) [2].

The textbook view of the link between ABC transporter function and structure invokes a nucleotide-dependent conformational switch, consistent with the idea of alternating access to the inside and outside of

Abbreviations
ABC, ATP-binding cassette; cryo-EM, cryo-electron microscopy; IF state, inward-facing state; OF state, outward-facing state; TAP, transporter associated with antigen presentation; WT, wild-type.

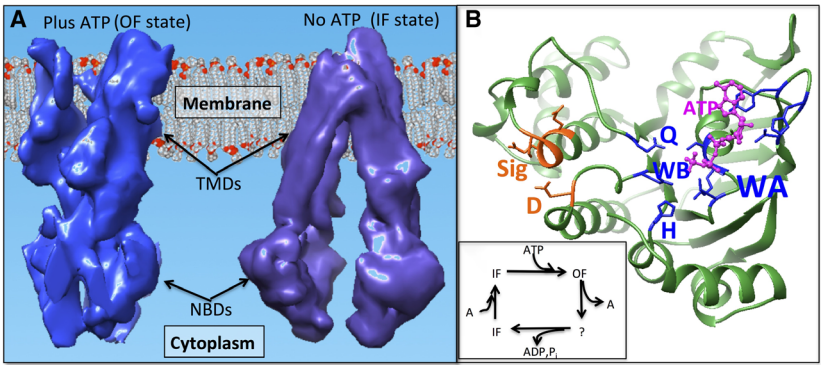

Fig. 1. Textbook views of ABC transporter function. (A) Transmembrane domains are embedded in the lipid bilayer (spacefill representation of the lipids), whilst the heart-shaped nucleotide-binding domains are inside the cytoplasm. Early structures in the presence of ATP (or nonhydrolysable versions of it) were OF (blue) with the NBDs dimerized, whilst those without ATP were IF (purple) and with NBDs separated. This was entirely in accord with Jardetzy's alternating access hypothesis; however, more recent structures have begun to question the simplicity of this model. (B) Conserved regions in the prototypical NBD of HisP as viewed from the NBD–NBD dimer interface. The *cis* residues interacting with the bound ATP molecule (magenta) are coloured blue and include the Walker A (WA), Walker B (WB), Q loop (Q) and His loop (H). The *trans* residues that bind the ATP on the opposing NBD are shown in orange and include the D-loop (D) and signature sequence (Sig). (C) shows a simplistic scheme for the transport cycle for an exporter, where A is the transported substrate (or allocrite). For importers, the scheme can also be applied, but for the allocrites the entry/exit arrows will be reversed. The outward-occluded state is not included and uncertainties are indicated by the question mark.

the cell as originally proposed by Jardetzky, to explain transporter function in general [3] (Fig. 1). It therefore follows that NBD dimerization and dissociation are pivotal stages of the substrate translocation cycle of an ABC transporter. Intriguingly, however, this apparent consensus model for ABC transporter function was not arrived at smoothly. Likewise, the precise nature of NBD association and release remains unclear, including occasionally fierce debates as to whether NBDs ever truly dissociate in the context of a full-length ABC transporter. Finally, the nature of the hydrolysis step and the subsequent signal propagation throughout the transporter domains remain under debate [4].

The two structures shown in Fig. 1 [5,6] are for the same eukaryotic ABC protein, P-glycoprotein (Pgp, ABCB1) that we now know to be structurally representative of three of the human ABC transporter subgroups (ABCB, ABCC, ABCD) as well as many bacterial transporters, including the lipid A transporter MsbA or the *Staphylococcus aureus* protein Sav1866 [1,7]. The first low-resolution structure of Pgp showed the transporter in an outward-facing (OF) conformation with associated NBDs (Fig. 1A, left) and was produced from two-dimensional crystals of the Chinese hamster protein studied by cryo-electron microscopy (cryo-EM) in 2005 [5]. The first inward-facing (IF) high-resolution structure of mouse ABCB1 with fully separated NBDs (Fig. 1A, right) was obtained by X-ray crystallography of three-dimensional crystals 4 years later [6]. Since then, many more Pgp structures have been added to the mix, both in isolation, in the presence of ligands/inhibitors and/or nucleotides, for example [8–10].

For the bacterial transporters, the crystal structure of the ABC exporter Sav1866 in complex with ADP was reported in 2006 [11]. Here, the TMDs adopt an outward open state, whilst the NBDs are in close contact. As the Sav1866 structure presented the first reliable structural view of a full-length ABC exporter, it became the gold standard for the generation of a vast number of homology models of human ABC transporters. Its publication was preceded by the first structure of a bacterial importer, the vitamin B12 transporter BtuCD [12]. In hindsight, it is tempting to speculate where the field would have gone, if only this latter structure had been available for homology modelling and what we have missed or where we have been led astray so far because unique structures of specific transporters and/or states are still amiss. Since these first careful steps into ABC transporter structural biology, at least one further state has been identified: the outward-occluded state where the nucleotide binding domains are close together but the transmembrane domains have not opened up to the outside [13,14].

Additionally, two separate classes of ABC transporters have been discovered that add to the complexity of the emerging picture of ABC transporter function and structural diversity: first, the bacterial energy-coupling factor (ECF) transporters operate

with an elevator or swivel type of alternating access mechanism rather than the outward-to-inward hinge-like motion shown in Fig. 1A [15]. These transporters appear to have given rise to the plant ABCI transporters [7]. Second, the bacterial 'mechanotransducer' ABC transporters (which may have given rise to the ABCA, ABCG and ABCH families [7]) do not transfer substrates across the cell membrane, but rather they seem to transduce a mechanical force into the periplasm which is then coupled to the movement of substances out of the periplasm into the external medium [16]. In all these cases, the NBDs are crucial for providing the energy to fuel these macromolecular machines. However and somewhat frustratingly, details on the mechanical coupling between NBD and TMD, the number of ATP hydrolysed per cycle, the allosteric consequences of substrate interaction within the TMDs, the precise order of events in the ABC transporter catalytic cycle and whether NBDs fully or only partially separate during these steps remain unclear.

Models for ABC transporter–transport cycles with complete NBD separation (ATP switch [17], tweezers [18] or processive clamp [19]) as well as those without separation (alternating site [20], constant contact [21], nucleotide occlusion [22]) have been put forward (reviewed in, e.g., [23]). It is possible that the details of these mechanisms depend on the specific ABC transporter under scrutiny, but fundamental questions such as whether complete NBD separation is a physiologically relevant state (or indeed the symmetrical ATP-bound sandwich dimer), as well as the biophysical properties governing NBD dimerization and (partial) separation, remain unanswered. In this review, we mainly focus on homodimeric half transporters and eukaryotic full transporters, but there is emerging evidence that the situation of the NBD association/dissociation is also not an open-and-shut case for heterodimeric ABC transporters. We also purposefully ignored the role of substrates for NBD closure or the details of stimulated ATPase activity. The absence of such considerations in this review is not intended to reflect their lack of importance but rather is for reasons of space and simplicity.

NBD structures – history and current challenges

In contrast to the complexity of the TMDs, the diversity in substrates and lipid environment of ABC transporters, it may be tempting (yet unfortunately misleading) to assume that the NBD, the most structurally and sequentially conserved part of the ABC protein superfamily, is easier to understand. All NBDs consist of a RecA-like domain that contains the Walker A and Walker B motifs important for ATP binding and hydrolysis and an α-helical domain that harbours the ABC signature motif, or C-loop [24,25]. The presence of these three motifs designates a protein as a member of the ABC superfamily. Whilst their structures are highly conserved, the position of the NBDs in the ABC transporter polypeptide chain can vary. In ABCG and ABCH transporters, the NBD is N-terminal to the TMD. In ABCA, ABCB, ABCC and ABCD transporters, it is located on the C terminus. Additionally, ABCB, ABCD, ABCG and ABCH transporters can consist of half transporters (that may homo- or hetero-dimerize), or of full-transporters such as Pgp (ABCB1), where all domains are present on a single polypeptide chain. In bacterial ABC transporters, additional domain organizations with separated NBDs are also common.

Based on the difficulties of working with and obtaining structures of membrane proteins [26], it is not surprising that the first high-resolution structural data for an ABC transporter were obtained for an isolated, soluble NBD, HisP, rather than a full-length transporter [27]. As outlined below, this structure did not provide an immediate, clear explanation of NBD function. The structure of the HisP NBD with bound nucleotide displayed a crystal packing arrangement that was initially interpreted as showing the physiologically relevant dimer [27]. However, the nucleotides in the structure were distant from the signature motif that is a conserved feature of all ABC proteins [25]. Subsequent thought was that the HisP crystal arrangement was probably a product of the crystal contacts rather than representative of the physiological NBD dimer. Experimentally, dimerization of the soluble HisP NBDs (driven by titration to high protein concentrations) led to a ~ 3-fold stimulation of ATPase activity as demonstrated by Ames and co-workers [28]. Jones and George proposed the structural basis for this finding via a simple rearrangement of the HisP structure that allowed the conserved ATP binding sequences within the NBDs to concertedly bind the nucleotide; that is, each binding site was formed by the Walker A and B motifs from one NBD monomer and the signature sequence from the opposing NBD monomer [29]. This so-called 'sandwich dimer' was later observed for a nontransporting NBD dimer, Rad50 [30] as well as for an NBD of unknown function, Mj0796 [19]. It has since then been found in subsequent structures for (full-length) ABC transporters and also in NBD dimers that have disparate nontransporter functions (such as the human ABCE and ABCF families). The

ECF transporters as a possible exception to this rule are considered later (see below).

That the initial structural study with HisP failed to generate a physiological dimer, despite the presence of nucleotide, was not an example of *sod legem scriptor*, but may instead reflect the possibility that dimerization of the NBDs is not intrinsically triggered by the binding of ATP. Rather it may depend on events associated with the TMDs such as substrate binding (see below). In return, as predicted previously [31], this would suggest that dimerization of isolated NBDs is generally an unfavourable event with a ΔG close to zero for dimer formation even in the presence of ATP. Such considerations, whilst somewhat controversial and based only on a small number of available NBD structures, did have the merit of being compatible with the need to avoid unregulated ATP hydrolysis within the cell. Now, with many more isolated NBD as well as full-length ABC transporter structures and functional data available, the main conclusion that NBD dimerization is not typically energetically favourable still seems to hold.

To dimerize or not to dimerize? Studies on isolated ABC transporter NBDs

Despite their important contributions to ABC transporter structural biology, recently the study of isolated NBDs has not been deemed a particularly rewarding endeavour (i.e. with regard to the debatable impact factor metric of the resulting publications). Reasons for this can be traced back to the development of more advanced expression systems for full-length transporters, including eukaryotic ones, to an increase in the availability and access to advanced technology such as cryo-EM that allows full-length or near full-length transporters ABC proteins to be visualized, sometimes even under near native conditions (see, e.g., contribution by Januliene & Möller, this issue). However, despite the undisputable importance of working with full-length transporters, for example for structural purposes, it needs to be pointed out that many ABC transporter structures designated 'full-length' proteins suffer from mediocre resolution, absence of loops or otherwise flexible regions and still do not reveal details about, for example transition rates of a system in solution. Furthermore, it is striking that many central questions specifically concerning the NBD and its interaction with nucleotides, intradomain cross-talk between conserved NBD motifs, the structural and dynamic propagation of the ATP binding event throughout the *cis*-NBD and the parameters governing NBD dimerization and dissociation remain fiercely debated today, more than 40 years after the discovery of P-glycoprotein and more than 30 years after the identification of the NBD as the unifying structural and sequential motif of an ABC protein. There is thus still much to gain from studying isolated NBDs and then comparing their properties with NBDs in full-length transporters.

The charge distribution of the NBD interface is crucial for NBD dimerization via charge complementation. ATP carries four negative charges that counteract the repellent forces of the positively charged Walker A and the C-loop. However, in the absence of a TMD or a connecting linker (see below), in the majority of NBDs that have been studied, ATP binding seems insufficient to efficiently promote NBD dimerization in solution, even at high protein concentrations as pointed out above. Consequently, isolated NBDs frequently show no or only very low ATPase activity [28,32].

Nucleotide binding domain dimers of isolated domains have, however, been observed in crystal structures or in solution when nucleotide was present and where the normal protein properties were modified, for example by generating the catalytically inactive mutant replacing the conserved Walker B glutamate by glutamine (E/Q mutation). For instance, one of the first NBD dimer structures was obtained for Mj0796 E171Q, an NBD present in a thermophilic bacterium (*Methanocaldococcus janaschii*) [19]. In a complementary series of experiments with the [wild-type (WT)] purified NBD using luminescence to detect NBD separation distances, Altenberg and co-workers showed that the isolated NBD operated in a way that was consistent with the idea of two ATP molecules driving NBD dimerization, whilst ATP hydrolysis at either active site would cause complete NBD dissociation [33,34]. However, the role of the TMDs for this system remains a mystery: we note that Mj0796 (NBD) is closely associated on the genome with Mj0797 that shows striking homology to the transmembrane and periplasmic portions of the mechanotransducer, MacB. Hence, there is circumstantial evidence that Mj0796/Mj0797 may be a mechanotransducer rather than a prototypical ABC transporter as originally assumed. From structural studies, mechanotransducer NBD–NBD separation has been found to change little [35] and large inward-to-outward conformational changes of the TMDs are not observed. However, there are large changes in the periplasmic (mechanotransducing) domains. A comparison of ATPγS-bound MacB with the nucleotide-free form of the same protein [16] (and the closely related protein Spr0694-0695[36]) shows only a small change in the

respective NBD separations and little change in the membrane-spanning portions (rmsd ~ 3.1 Å across 353/413 atom pairs within 6 Å). However, the periplasmic domains (~ 190/245 atom pairs are > 6Å distant) change greatly upon nucleotide binding, as perhaps would be expected as they represent the main mechanotransducing portions of the proteins.

A separately expressed NBD of an ABC exporter, HlyB, has also been studied extensively by biochemical and biophysical approaches [37–39] as well as by crystallography. After removal of the TMDs, catalytic inactivation of the NBD (via a mutation in the H-loop, H662A) was needed to generate the stable dimeric form for crystallization [40,41]. The H662A mutation in HlyB might also be deemed to alter the charge at the dimer interface since the pH of the final cryo-buffer for mounting the crystals was 6.2, that is close to the pKa for this residue. The HlyB NBD shows a strong pH dependence in its ATPase activity, with a maximum around pH 7. Similarly, and akin to HisP, it shows a concentration-dependent variation of ATPase activity, indicative of the need for dimerization [39]. Although no full-length structure for this transporter is currently available, a structure for a functional and structural homolog in the IF state (PCAT1) has been described, and this structure is similar to ABCB1 (Fig. 1) with long transmembrane α-helices, which show a domain swap for the second intracellular loop (ICL) [42]. Even in the absence of ATP, the two NBDs of PCAT1 are in contact through the tips of their C-terminal helices and with the Walker A and H-loop from one NBD facing the D-loop of the opposing NBD in a 'semi-open state' [43]. The NBDs fully close upon nucleotide binding, but to obtain this state in a crystal, again the E/Q mutation had to be introduced and the nonhydrolysable ATP analogue ATPγS was used. The requirement for catalytic inactivation in so many structures seems to suggest that continuous ATP hydrolysis at alternating sites may be inconsistent with stable dimer formation.

However, there seem to be rare cases where NBDs readily dimerize with the help of ATP, even in the absence of mutations or auxiliary domains or linkers. Examples are the nonphysiological NBD1 dimers from ABCC7/CFTR in the absence of a CFTR-NBD-specific regulatory insert [44] or NBD1 from the heterodimeric transporter associated with antigen processing (TAP1/2) [45]. In the case of the TAP NBD, this effect is even species-dependent. Whilst the isolated NBD from rat TAP1 forms ATPase competent homodimers [45–48], the isolated homologous human protein does not [47]. ATP-dependent rat TAP NBD1 dimerization can be abrogated by mutating residues next to the conserved D-loop in the 'D-helix' [45,47]. Indeed, the D-loop (consensus sequence SALD) has been described previously to play important roles in NBD–NBD communication and NBD dimerization, for coupling ATP hydrolysis with substrate transport and, in the case of TAP, for the unidirectionality of transport [40,49–53]. Importantly, the opposite experiment, introducing a set of five D-helix residues from dimerization-competent rat TAP NBD1 into human NBD1 to allow to the formation of ATP-inducible human TAP NBD1 dimers with ATPase activity identified the D-helix as an important element for NBD association across the canonical sandwich dimer interface [45].

This indicates that forming a NBD dimer in solution is not a difficult task for nature *per se*but rather that she spent quite a bit of effort fine-tuning the interface to allow the NBDs to associate in the presence of ATP and, potentially even more importantly, to part relatively easily. NBD dimerization needs to be reversible after ATP hydrolysis, ADP and phosphate release, and thus, the free energy for dissociation of the dimer should not be too large and positive. Examination of the crystallographic unit cell of the rat TAP1 homodimeric NBD in the sandwich configuration [45–48] shows that the NBDs are on the cusp of dimerization: the ΔG value for dissociation is predicted by the European Bioinformatics Institute PISA server to be only +4 kcal·mol^{-1} for one dimer in the asymmetric unit, whilst the other dimer in the asymmetric unit is a meagre +0.8 kcal·mol^{-1}.

ABC importers seem to adopt a different strategy for stable NBD association that relies on an additional C-terminal domain fused to the NBDs that forms an extensive dimeric interface with the equivalent domain of the opposing monomer. These auxiliary domains can interact with the respective transporter substrate and thus provide a means for 'trans-inhibition' to avoid substrate over-accumulation [54–56], or in the case of the particularly well-studied maltose importer $MalFGK_2$ are important for carbon catabolite repression by interacting with regulatory proteins [57]. The isolated NBDs (MalK) of the maltose transporter were investigated in various states using fluorescence probes [58] or crystallography [18]. MalK NBDs remain associated with each other in the available structures, probably due to the presence of additional C-terminal regulatory domains. In the context of the full-length transporter in the absence of nucleotides, complete canonical NBD separation is observed, but the C-terminal domains nonetheless tether the NBDs in close proximity [59]. Hence, whilst the maltose transporter NBDs rock inwards and outwards upon ATP binding and hydrolysis (respectively), the regulatory domains hardly change their relative orientations [18,60].

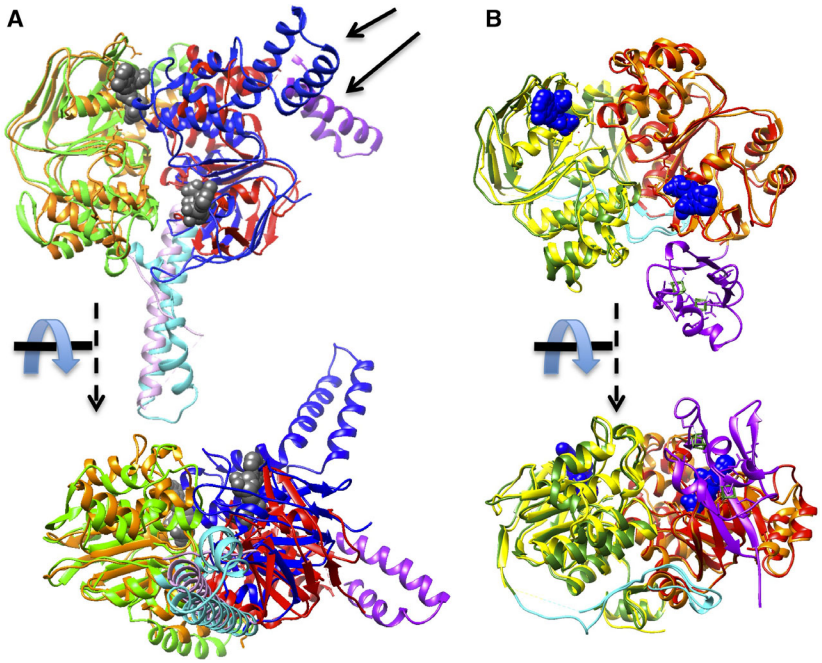

Fig. 2. NBD dimers without TMDs. (A) Alignment of ABCF1 NBD1 and NBD2 (5ZXD, red, orange, respectively) with the bacterial EttA NBDs (3J5S, dark blue, green). The NBD1-NBD2 linker is shown in pink (ABCF1) and cyan (EttA). Part of the former linker is disordered. The grey spacefill atoms represent ATP in ABCF1. On the right, the ribosome-interacting insertion in the helical subdomain is indicated (arrows). For ABCF1, the insertion is shown in purple. Two orthogonal views are shown, the top view is roughly parallel to the two-fold pseudosymmetry axis of EttA. An outward rocking motion (opening) of the ABCF1 NBDs is manifested as a ~ 65° rotation of NBD1 (red) compared to EttA (dark blue) in the lower view. (B) Alignment of the bacterial ABCE1 ortholog structures with PDBID 3BK7 and 3OZX. Each shows a relatively short, but (mostly) structured part of the polypeptide chain (cyan) that links NBD1 (orange, red) with NBD2 (green, yellow). Nucleotides (ADP) are shown in spacefill (dark blue). The purple extension at the N terminus is an iron-sulphur (FeS) domain that was not present in a current structural analysis (3OZX, to be published). Note that the NBDs have the rocked-open configuration, as also seen for ABCF1 in panel A.

Thus, for the majority of *in vitro* studies, mutations in the NBD interface to promote dimer formation seem to be required, whilst *in vivo*, auxiliary domains on the NBD C terminus as for MalK, the TMDs in integral membrane ABC transporters, or linkers between the NBDs (in the case of ABCE and ABCF proteins, see below) enable NBD dimerization. The vast number of examples for isolated NBDs refusing to dimerize in solution in the absence of linkers, mutations or the TMDs underscores the notion that this finding is not merely a laboratory artefact but rather reflects an important intrinsic biophysical property of these proteins that can be exploited in the context of full-length proteins to regulate and fine-tune activity. The apparent reluctance of isolated NBDs to dimerize may also have implications for our take on 'futile hydrolysis', that is basal ATPase activity in the absence of substrates frequently observed in *in vitro* systems, in particular for ABC transporters with some substrate promiscuity such as the 'multidrug resistance ABC transporters'. On the one hand, this phenomenon could simply reflect the fact that for such systems, true basal activity is never actually measured but rather an uncoupling between TMD and NBD or a stimulation via lipid or detergent molecules takes place. On the other hand, even this interpretation critically underscores the importance of the transporter environment and the TMD for the proper regulation of ATP interaction and hydrolysis. Thus futile hydrolysis, if it indeed exists, is presumably not entirely the NBD's 'fault'. In contrast, it seems that in general the NBD is doing everything it can to keep its distance from its partner even in the presence of ATP whilst TMDs, lipid environment or linkers (see below) are counteracting this tendency to ultimately give rise to a finely tuned, highly efficient molecular machine.

The role of the TMDs for NBD dimerization

Whilst the preceding section reviews nucleotide-dependent dimerization from the perspective of the NBDs, it is also important to consider that the lipid-embedded

TMDs are likely to play a major role in modulating this process. The free energy for dissociation for the TMD-TMD dimer is generally high and positive [31], and thus, the TMDs form the stable core of the transporter complex and probably make up the assembly platform in bacterial systems where the transporter is composed of four separate subunits. For most transporters, both of pro- and of eukaryotic origin, it is clear that the TMDs can hold the NBDs in a position ready to dimerize. Complete dissociation of the NBDs from the TMDs and thus in principle, free rotation along the linker peptide chain, has been discussed for bacterial half transporters in the past and extensive NBD dynamics have been observed experimentally [61–63]. However, a complete detachment of the NBDs from the TMDs as an intermediate of the transport cycle seems rather unlikely based on the available structures where, even in outward open transporter structures with fully separated NBDs, the NBDs always interact with the TMD coupling helices and face each other in a dimerization-competent manner. Occasionally, the NBDs are displaced sideways, indicating a complex multidimensional rotation that is required to transgress from an inward open, nucleotide free to an occluded or outward open, nucleotide-bound state [62].

The important role of the TMD for NBD dimerization is further supported by the observation that substrates can increase the ATPase activity of transporters and that ATP binding and hydrolysis are sufficient to trigger long-range conformational changes in the TMDs. Such features of bidirectional domain communication may be a feature common to all ABC transporter families although this remains to be experimentally demonstrated for, for example, the mechanotransducing ABC transporters. It may, however, not just be the relative NBD positioning by the TMD that is crucial but also a direct impact of the TMD on the intrinsic NBD dynamics. For instance, parts of the NBD of a heterodimeric ABC transporter, specifically the D-loop, were observed to be disordered in crystal structures in isolation, but not in the context of the full-length transporter [64].

Additionally, it is becoming clear that not just the TMDs but also the transporter environment, the lipid bilayer, plays a crucial role [65]. For instance, MsbA exists mainly in an inward open conformation with widely separated NBDs in detergent as observed both in deposited structures [66–68] and from biophysical measurements of NBD separation [69,70]. In contrast, placing the protein in liposomes or nanodiscs leads to the population of both inward and outward open states and in the lipid environment, the NBDs are in closer proximity to each other compared to what is observed in detergent, even without the presence of ATP [69,70]. Consequently, lipids have a stimulating effect on MsbA's basal ATPase activity, something that has been observed for many ABC transporters [71,72]. Whilst already indicating that the lipid environment is an important driver or enabler of ABC transporter NBD interaction, it is important to bear in mind that these model systems still miss many crucial components, such as lipid asymmetry or the physiological lipid composition of a natural system.

The role of linkers in NBD dimerization

NBD-TMD linker domains in ABC transporter proteins were reviewed recently [7,73]. For some full ABC transporters (with all four domains on a single polypeptide chain), disordered linking regions of 50–200 residues in length tether the end of NBD1 to the start of TMD2. It seems likely that interposition of the linker polypeptide between the NBDs may regulate NBD dimerization via steric hindrance, perhaps explaining the difficulty in forming the OF state for these transporter types [7]. Likewise, within the 'reverse topology' of the ABCG family, such a linker of about 60–70 residues in length is located between TMD1 and NBD2 (see, e.g., the Pdr5p yeast ABCG family transporter) and may again crucially interfere with stabilizing an NBD-dimerized/OF state for structural studies.

There are also domain linkers in the ABCE and ABCF families (see also contribution by Boel and coworkers, this special issue), but in contrast to the transporter families, these appear to have evolved to aid NBD dimer formation. Figure 2A shows the structures of a bacterial NBD dimer, EttA, and the human ABCF1 protein. These two proteins align well, structurally, and in both cases, there is a coiled linker region that connects between the end of one NBD and the start of the C-terminal NBD. The linkers (cyan, Fig. 2A) form a hairpin-like structure consisting of two α-helices with a right-handed super-helical twist. It is tempting to speculate that the linker would apply a spring-like function so that, even in the crowded conditions of the cell, the NBDs are able to readily dimerize. The binding and hydrolysis of ATP in the ABCF family is associated with reversible binding of the NBD dimer to the ribosome, and this, in turn, is linked to translation regulation and in some cases, antibiotic resistance [74,75]. Of note, although ABCF1 has also recently been proposed to moonlight as an E2 ubiquitin ligase [76], the critical Cys residue identified

(C647 in mouse, C657 in humans, C357 in the renumbered PDB file) as well as other loosely conserved residues (e.g. P615 in mouse) are buried in the main β-sheet subdomain of the NBD and seem unlikely to be able to form a E2 ubiquitin ligase-like site without major rearrangement of the ABCF1 conformation.

ABCE proteins are highly conserved across species but usually a single *abce1* gene is present, as in humans. They are associated with translation termination and ribosome recycling [77] although other functions may exist [78]. For this family, an entirely different linker region is observed which resembles a clamp, however with the same tethering effect as seen for the ABCF linkers. The ABCE1 NBD dimer linker is a relatively short, but nevertheless structured part of the polypeptide chain (cyan, Fig. 2B), and extends over the surface of the C-terminal NBD like a clamping arm. A similar NBD dimer linker is also present in the eEF3 elongation factor protein, an ABCF family member, involved in tRNA release from the ribosome. Here, the NBD–NBD association appears to be non-canonical in the apo state when dissociated from the ribosome [79]. A typical NBD–NBD sandwich dimer with nucleotide bound is found in the cryo-EM structure of ribosome-bound eEF3, implying a major rotation and rearrangement of the NBDs upon ribosome association [79,80]. The linker between the first and second NBDs in eEF3 is similar to the ABCE-type linker. It is structured, about 30 residues in length, and wraps around the surface of the second NBD. Remarkably, the linker's position on the surface of the NBD2 does not change much, despite the complete 180° rotation of the domain when forming the canonical dimer. Despite the fact that ABCE and ABCF proteins do not contain TMDs, their NBDs are still tethered via linkers thus again giving credence to the notion that unsupported NBD dimerization is not an easy undertaking.

Other NBD–NBD only dimers exist that are involved in DNA repair [81], such as the UvrA, MutS, Rad50 and RecF proteins. UvrA has a clamp-like linker joining the N- and C-terminal NBD that is very similar to the ABCE family linker [82]. MutS [83], Rad50 [84] and RecF [85], however, have no linker, and the NBD must find its partner in the cell in order to dimerize. Nonetheless, these DNA repair proteins have large DNA-binding extensions inserted into the helical subdomain of the NBD (e.g. Rad50, RecF) or fused at the N terminus (e.g. MutS). It seems likely that for these proteins, DNA acts as a template for assembly and dimerization of the NBDs, with rapid 1D diffusion of the proteins along the DNA molecule facilitating this process. Moreover, these ABC proteins usually work together with other DNA repair proteins, such as UvrB, Mre11, RecO, RecR or RecA, hence forming parts of larger DNA/protein complexes where dimerization may be mediated by other proteins in the complex.

The question of whether linkers in the ABCE and ABCF families are in any way related (through evolution, structure or function) to each other as well as to linkers in the other ABC families remains to be explored. In transporter ABC families, the NBD1-TMD2 linker has a length that is similar to the ABCF/EttA NBD1-NBD2 linker and coincidentally, a linker that could take up a helical hairpin conformation, was proposed for ABCB1, based on weak homology to EsxB, a secreted peptide [7]. In general, physical tethering of the NBDs or taking advantage of cellular assembly platforms such as DNA and/or other proteins therefore seems to be a way in which the ABCE and ABCF families overcome the problem of assembly and dimerization in the complex and crowded conditions of the cell in the absence of a TMD.

NBD interaction and separation in full-length ABC transporters – some examples

As mentioned above, in structures of full-length ABC transporters, stabilization of the OF, NBD-dimerized state for structural studies is difficult, and requires mutations in most cases. For example, to trap the ABCG2 dimer in the OF state, the catalytically inactive E211Q mutant as well as ATP were needed [86]. However, the fact that these two key levers allowed the OF state to be stably populated also lets one surmise that such an NBD dimer, once formed, will be stabilized by additional interactions between ABCG2-specific segments. For example, a region of interest is an extension to the loop prior to the α-helix housing the canonical signature sequence (Fig. 3). This loop/extension is interacting with the elbow helix of its own (cognate) TMD in the IF state, but in the OF structure it forms a contact with the first ICL of the opposing TMD [86,87]. The F182 residue in the loop makes contact with K432 and K433. A serine residue in the second ICL of the opposing TMD, S532, also appears to be able to interact with the F182 residue. This loop extension is also present in ABCG5/G8 although the phenylalanine residue is replaced by tyrosine. In contrast, ABCG1 and ABCG4 do not show this loop extension in their amino acid sequences, and are more typical of NBDs of other ABC transporters. At the C terminus of the NBD in ABCG2 in the OF state [87],

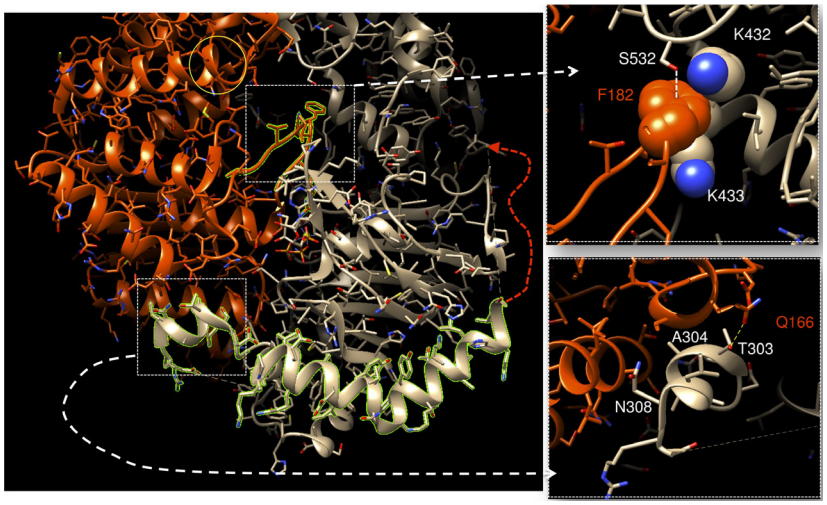

Fig. 3. Stabilization of the NBD dimer in ABCG2. (A) Dimerized NBDs in ABCG2 (orange/cream) show a domain-swapping extension of the C terminus of the NBD (green highlighted cream section) which interacts with the α-helical subdomain from the opposing NBD before reaching back around its cognate NBD surface and forming the start of the disordered linker to the TMD (dashed line). The lower inset shows the packing of the residues in this region, with T303 potentially forming an H-bond with Q166. A loop emerging from one NBD that is just prior to the signature motif (green highlighted orange section, arrow) extends up to form contacts with the connecting ICL from the TMD of the opposing monomer. F182 inserts between R452, R453 and may form a lone-pair:π interaction (dashed line) with S532 in the opposing monomer (inset, close packing residues are shown in spacefill). In the IF ABCG2 structure, this loop is interacting with the elbow of the elbow helix at the start of its cognate TMD (yellow circle). This insertion is conserved in ABCG5/G8.

there is a short helical extension that crosses over and forms a hydrophobic contact with the opposing NBD. This extension is then connected to the start of the linker polypeptide that transits over the surface of its cognate NBD as a helical element. There is some similarity between this arrangement and the 'clamp' linker in the ABCE family (Fig. 2B), but no sequence similarity appears to be present and the arm of the clamp is around a different side of the NBD in ABCG2. In the IF state, the interacting helix is disordered and the extension of ~ 10 residues may not be long enough to cross over to the opposing NBD. A more detailed exposition of the ABCG transporters can be found in this issue (Review by E. Lee *et al.*, this special issue).

Other ABC transporters in the OF state may use a similar mechanism to stabilize the dimerization of the NBDs via C-terminal linker extensions. For example, the 2017 structural deposition for ABCB8, a mitochondrial ABC transporter trapped in the OF state (PDBID 5OCH), has a helical extension at the NBD C terminus, which also crosses over and interacts with the equivalent extension from the opposing NBD. The last ~ 15 residues of the C terminus are missing in the structure. In this case, the interactions seem to be via an alanine zipper motif, with hydrophobic and Van der Waals interactions predominating. Presumably, these interactions are weak because they are only modelled in one of the four ABCB8 homodimers forming the asymmetric unit. This work remains to be published, but it is very interesting to note that in this case, no catalytic inactivation of the protein was needed to form the OF state (Faust *et al.*, Structural Genomics Consortium). However, the protein still was modified by a truncation of the N-terminal ~ 110 residues, similar to the ABCB10 structural exploration [88]. Recently deposited, but yet unpublished structures for the *Bacillus subtilis* transporter BmrA in the OF state (PDBIDs 6R72 and 6R81), which, as observed for the majority of other (homodimeric) ABC transporters, required the mutation of the catalytic glutamate (E504A), also show interactions of the C-terminal extensions of the NBDs in a similar fashion to ABCB8. Overall, C-terminal NBD zippering seems to be a very common feature of dimeric NBDs both in homo- and heterodimeric ABC transporters, which had been noted early on in, for example, the structure of the HlyB NBD dimer [40]. However, depending on the transporter type, zippering can be associated with different intermediates of the ABC transporter catalytic cycle. For instance, in the structure of the heterodimeric thermophilic ABC transporter TmrAB, these interactions are associated with an inward open state of the TMDs [89]. Crosslinking experiments indicated that the TmrAB C-terminal helices undergo rearrangements during the catalytic cycle and their deletion significantly reduced substrate translocation.

It also remains unclear whether these C-terminal helices behave differently when comparing heterodimeric (structural rearrangements but retention of contact?) and homodimeric (possibly full dissociation?) ABC transporters.

Structural/biophysical studies – challenges and limitations

Because of the limitations and narrow requirements for structural studies, our understanding of any ABC transporter may be biased: Structural data will usually provide a limited number of snapshots of what may be a highly mobile protein. For example, protein crystal formation is usually only possible with one (or a limited number of) conformational state(s) and only rarely do crystal asymmetric units contain polypeptide chains with significantly differing conformations. Cryo-EM analysis of single particles of transporters should allow multiple conformations to be identified, but studies often focus on just one 3D class (usually the one showing the best final resolution). Any 3D conformations that show low-resolution or weak intermolecular interactions (manifested as transient aggregates) will typically be discarded during cryo-EM single particle analysis [26] although recently eight structures of the same thermophilic ABC transporter were presented in different catalytic states by cryo-EM [90]. Nonetheless, such studies are still limited by the need to infer motional trajectories, which need to be obtained from complementary studies and the absence of transition rates. Hence, and as the ABC transporter field in particular is well aware of, data from biophysical studies are important to balance our critical understanding of structural studies of ABC transporters. An example of this is HisP, as discussed earlier, where the sole interpretation of the initial crystal structure would not have led to a reasonable model of the *in vivo* situation [28,29,35].

In recent years, the biophysical toolkit has been successfully expanded from NBDs to full-length transporters. Available static structures in combination with approaches to probe distances between reporters attached to cysteine residues (e.g. EPR, FRET or LRET) for instance indicate that in the absence of ATP, ABC transporters can adopt multiple conformations including those with fully separated NBDs and that these conformations are modulated by substrate and ATP binding or hydrolysis [13,91–101]. Of course, such studies have the caveat that they predominantly focus on bacterial transporters due to their easier availability in high amounts although examples for eukaryotic ABCB1 similarly indicate that the apo state with dissociated NBDs exists for this transporter in solution as well [99]. Indeed, and in agreement with the large majority of the structural database for ABCB1, the IF configuration, with separated NBDs, was observed in the majority of experimental conditions, even in the presence of a nonhydrolysable ATP analogue [99,102]. Obtaining the outward open conformation of ABCB1 thus seems to be no mean feat. From a recent EPR study on ABCB1, apo-like distances for the NBDs were observed in the presence of ATP/Mg^{2+} and it was postulated that in this transporter, the OF state is very short-lived [99] and hence would provide a kinetic hypothesis as to why the outward open state is so infrequently caught in structural studies. The same study found that, when trapped with vanadate in a posthydrolytic state, ABCB1 favoured the OF conformation [99], which was not in agreement with the cryo-EM data from two separate studies [8,93]. It remains to be seen where these differences stem from, but overall these data support the idea that formation of the NBD sandwich dimer and the OF state is quite difficult in Pgp. Similar observations, as reflected in the recent glut of cryo-EM structures, are made for other eukaryotic ABC transporters (see, e.g., the 'grunt' model for CFTR/ABCC7 [103]).

Where the OF conformation has been generated and the structure elucidated, this has usually been done by stabilizing it using catalytic inactivation, often in combination with conformation-specific antibodies. Furthermore, there are now many studies of full-length transporters with nucleotides bound that are nevertheless in the IF state, the first example being human ABCB10 [88]. Unusually, multiple structures also exist for an amino acid transporter where nucleotide and substrate bound states are virtually identical (all within 0.2 Å RMSD) to the apo structure [104]. These more-or-less identical structures are all in the IF state, with NBD–NBD separation about 1 nm greater than for a sandwich dimer configuration. Hence, from structural studies, one could form the impression that, at least for eukaryotic transporters, the IF state predominates, even under the physiological conditions in the cell (at mM ATP concentrations). A caveat here is that the preponderance of the IF state could be an artefact of the isolation and *in vitro* experimental systems.

Interestingly, the situation seems to be different in bacterial homodimeric exporters. Here, ATP binding within the NBDs typically triggers the outward open conformation or in other cases an occluded state [13] in concert with an overall rigidification of the transporter as assessed by spectroscopic techniques such as NMR spectroscopy [32,100,101,105]. However, when adopting such assertions it is important to bear in

mind that, similar to available studies on eukaryotic ABC transporters, they may also be biased by a small subset of (perhaps) unrepresentative proteins.

The (possibly futile) search for a harmonized understanding of NBD motions

This article was originally conceived as a two-way debate, although as it developed, the two protagonists' positions became increasingly blurred, perhaps understandably. Hence, it was written as a more traditional review of the literature (and data depositions) relating to ABC proteins. Nevertheless, some elements of the original concept remain, and we hope that this has not torn the reader in different directions in a completely confusing manner. In these final two sections, we try to bring about some consensus, but we acknowledge that this is still a personal perspective and leave the reader to form their own opinion about the ideas presented.

ABC transporter research has, from the early days of venturing into the structural biology of these systems and inspired by the Jardetzky model of transport, striven towards a harmonious model of transporter function. Such a model, if applicable, should satisfy all peculiarities of different transporter families, their substrates and their organism of origin. However, to paraphrase Karl Popper, a theory that explains everything explains nothing. Indeed, the studies from recent years have demonstrated that ABC transporters may not all follow the same functional template and trying to mo-ld them into one can lead researchers astray. NBD (dis)association during the catalytic cycle is but one prime example of this.

As discussed above, it is generally agreed that nucleotide-dependent NBD dimerization is inherently associated with ABC protein function [14,17,73,106,107]. However, the details of how this comes to pass may vary quite significantly between transporters. Figure 4 shows an overview of the available information on the nucleotide-dependent conformational movements of the ABC proteins.

In the eukaryotic ABCE and F subfamilies as well as in the bacterial type I and II importers, the NBDs undergo a rocking motion (Fig. 4A). The same type of rocking motion of the NBDs is observed in the mechanotransducer ABC transporters, although here small changes in the NBDs and TMDs give rise to much larger motions in the periplasmic mechanotransducing domains. It has been proposed from comparisons of the topology of the transmembrane domains that the mechanotransducers gave rise to the ABCA, ABCG and ABCH subfamilies [7], although for these latter examples the rocking/opening of the NBDs is noticeably larger.

The NBDs of ABCB, ABCC and ABCD subfamilies of ABC transporters as well as the type I exporters in bacteria such as Sav1866 and MsbA undergo noticeably different types of motions (Fig. 4B). In these cases, a trend towards complete separation of the NBDs is observed with a 'swinging-arm' motion and with the centre of rotation located in the transmembrane domains. (Of note, the outward-occluded state is not depicted in Fig. 4B). To this point, a harmonious view of the ABC proteins holds, that is that the ATP-dependent closing and opening motions of the NBDs (despite some minor differences in the mechanistic details) are coupled with function and that the ATP-bound sandwich dimer is common to all these proteins.

There remains another ABC family that has not so far been discussed—the energy-coupling factor (ECF) transporters (which appear to have given rise to the ABCI subfamily in eukaryotic plants). These proteins are bacterial and archaeal nutrient, vitamin and metal importers. This subfamily is set apart from the remainder of the ABC transporters in that the NBDs move hardly at all (in the currently available structures [108–114]). Hence, the idea of global NBD motions giving rise to function (import of substances into the cell or organelle) is much harder to understand for these systems.

Energy-coupling factor transporters consist of an integral membrane transport coupling (T) domain, a cytosolic homo- or heterodimeric NBD pair and a membrane inserted substrate binding (S) component, which is able to bind substrates with exceptionally high affinity. Different S components may interact with the same T-domain/NBD platform [15]. Based on the available structures, the S components rotate, translate or 'topple' in the membrane upon binding to the ECF transporter. Substrate release from the 'toppled' S component is suggested to occur through a cleft formed by the interface of the S component and the NBD via the opening of a 'trapdoor' consisting of three loops within the S component [109–111]. It is unclear what exactly triggers the trapdoor opening. Comparison of the apo and ATP-bound states of the folate ECF transporter and the apo state of the vitamin B12 transporter showed hardly any global structural differences, nor specifically in the trapdoor within the S component [109]. However, since there was no sign of the substrate in both apo and nucleotide-bound structures [109–111,113,114], these data led to the conclusion that both structural states most

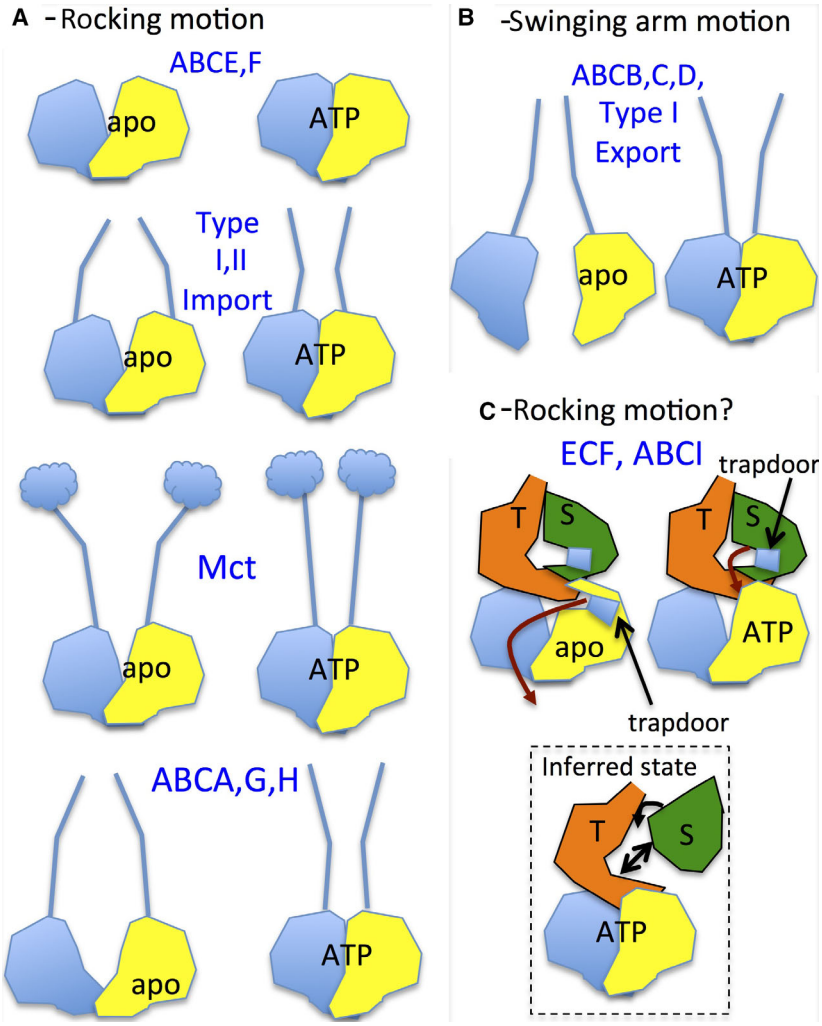

Fig. 4. NBD motions across the ABC family. (A) Rocking motion of the NBDs, with a pivot at the bottom is displayed for the ABCE & F families (and DNA repair enzymes), the type I and II bacterial importers, the mechanotransducers (Mct) and a larger rotation in the ABCA, G (and by supposition I) subfamilies. (B) In contrast the ABCB, C, D and type I bacterial exporters show a swinging-arm separation of the NBDs and the pivot point is in the TMDs. (C) The ECF importers and the plant ABCI subfamily (by supposition) pose a mystery as to their NBD motion/function (see main text), and this is the only group where the canonical NBD sandwich dimer has not (yet) been observed in a full transporter structure. ATP-bound and apo state structures are globally very similar, but local differences may be of significance, such as the opening of the 'NBD trapdoor' in the apo state. The red arrows indicate possible routes for substrate import. Note, for simplicity, the apo and ADP-bound states are conflated and the occluded state in panel B is omitted.

likely showed situations after substrate had already been released (i.e. the post-translocation state).

To date, and in contrast to all other ABC transporter subfamilies, no large scale movements for the NBDs have been observed so far in any of the available structures of ECF transporters regardless of nucleotide loading state or interaction with an S component [108–114] (Fig. 4C). The available structures show that NBD association is similar to states shown in Fig. 1A (rocked outwards NBDs), but that there is very little difference between nucleotide-bound and apo states (Fig. 4C) and no sandwich dimer is found in either state.

Although the fully closed NBD sandwich dimer has not been observed for the ECF transporters as of yet, the isolated NBDs in the absence of the TMD can be persuaded to form the canonical NBD sandwich dimer with AMPPNP [108] but not with ADP [112] or in the absence of nucleotides [17,115], potentially due to their extensive C-terminal helices (Fig. 5). Hence, there is an additional hypothetical conformational state (as shown in the dashed box, Fig. 4C) where the importer is in the sandwich dimer state and with a primed T component, ready to receive a freshly loaded S component partner. In this model that attempts to restore harmony, ATP binding and the formation of the sandwich dimer NBD could be associated with the process of the dissociation of the empty S component from the complex [108,109,116].

However, examination of the available ECF transporter structures also allows another possibility to be considered: Small, but consistent, differences were manifested between apo and nucleotide-bound states in the loop connecting the first and second β-strands of one of the NBDs. The start of this loop forms part of the binding site for ATP (e.g. Y12 in the riboflavin transporter NBD – PDBID 4ZIR) where an aromatic residue forms π-π stacking interactions with the

adenine ring of the nucleotide [112]. In the heterodimeric NBDs of the (complete with T and S components) folate transporter structure with AMPPNP, Y12 performs this function in one NBD and in the other it is the equivalent residue, F13 [109]. In the apo state structures, this loop has flipped completely outwards in the NBD containing Y12 (EcfA), whereas in the other NBD (EcfA') the loop remains in the same place as in the nucleotide-bound structures [109,112,114]. The NBD containing Y12 (EcfA) sits directly under the S component and also interacts with the first intracellular coupling helix of the T component. If this flexible loop represents a second trapdoor for substrate release from the transporter, it may be another way in which ATP hydrolysis could be coupled to import of substances into the cell, something that could be easily tested by mutagenesis. Interestingly, the EcfA NBD of the cobalt importer does not show the large outward flip of this loop, perhaps reflective of the much smaller substrate in this case [110].

Conclusions and the triumph of harmony over discord?

The association of the NBDs to form the sandwich dimer seems to be a common, probably universal, factor in the function of the ABC family of proteins. The large negative free energy inherent in ATP binding, hydrolysis and release of products, sometimes called the 'power stroke', appears to be linked to these conformational changes in the whole family. Intriguingly, structural and biophysical studies both on isolated NBDs and full-length transporters suggest that ATP binding generates the expected NBD-dimerized state, in particular for eukaryotic ABC transporters, much less often than expected (or is this just an observation bias?). It thus remains an important question whether the techniques that are employed, the systems that are being looked at or our own expectations of what we might find inherently bias our understanding of ABC transporters.

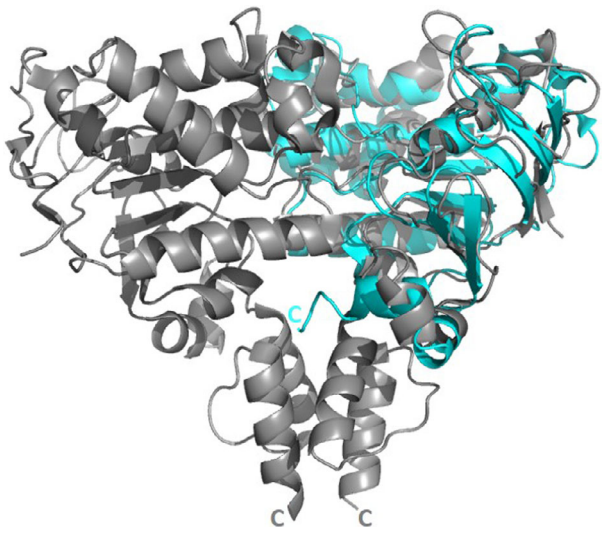

Fig. 5. Differences in C-terminal helices between different ABC transporter NBDs. Comparison of the NBD of an ABCB type transporter (cyan, Sav1866, pdb 2HYD) with that of an ECF transporter (grey, FolT, pdb 5JSZ) shows a different interaction in this region of the NBD which may potentially contribute to different dimer affinities for isolated NBDs. For both structures, resolution is complete to the last C-terminal residue.

A thought experiment may shed some light on this seeming conundrum: Consider that the free energy of ATP hydrolysis in these systems is coupled to changing the probability (P) of forming the required conformational state for substrate translocation. P is neither zero nor unity, as other factors such as thermal fluctuations will lead to a conformational distribution that precludes all transporters existing in the same state at the same time. Now, assume the probability of increasing the population of the OF state from $P = 0.001$ in the absence of ATP to $P = 0.1$. Addition of ATP thus leads to a significant (100x) increase in the population of this state. Nonetheless, the OF state remains significantly less populated than the IF state and under these circumstances, looking at such a system from a structural point of view, therefore will give the impression that the researcher is dealing with a transporter in the IF state although ATP was added. However, in terms of the capability of such a system to translocate a substrate, the change is highly significant (a ~ 5-fold effect on ΔG). Hence, these changes in probability/status may still be coupled to function such as overcoming a concentration gradient of a certain substrate (in the ABC transporters) or coupled to mechanical work (in the mechanotransducers and ABCE- and ABCF-type proteins). With this thermodynamic perspective in mind, the failure to produce the NBD sandwich dimer states in structural studies carried out in the presence of ATP may be a bit less perplexing. Moreover, keeping the NBD sandwich dimer to a miserly level, even in the presence of ATP, would seem to be a good way to avoid wasteful ATP hydrolysis in the cell. Almost ironically, it is the lack of dimerization in studies on isolated NBDs that make such points most conspicuous.

Indeed, as pointed out in this review, it is really difficult to 'convince' NBDs to dimerize. Nature, rather than optimizing the ATP-driven dimeric state seems to have put a number of checks and balances in place to

avoid NBD dimerization. We would argue that the fact that these systems do not 'want' to dimerize teaches us important lessons about our expectations and maybe our biases when studying ABC transporters. Of course, it can be argued that the full-length transporters in their native membranes are the physiologically relevant systems that should be studied. However, here we hope to have conveyed the idea that many of our current understandings about ABC transporters were only possible because we initially had isolated, monomeric NBDs available to study them on. And even now, decades into the process of elucidating ABC protein structure and function, many mysteries, including seemingly 'simple' questions such as 'How exactly is nucleotide binding propagated throughout the NBD (let alone to the TMD)' remain. We therefore argue that isolated NBDs still have to teach us quite a bit and that sometimes 'simplifying' a system allows to carry out much more detailed studies. But having said that, where to draw the line? All systems and all techniques come with their inherent limitations and biases. This could be the 'divide and conquer' approach of cleaving soluble domains to obtain first ideas about ligand binding or structures; the attachment of labels and/or size limitations (on the higher MW scale) for certain types of spectroscopy, the incorporation of lysozyme, generation of thermophilic mutants, cleavage of flexible regions or use of antibodies for crystallography; the size limitation (on the lower MW scale) and use of, for example, megabodies for cryo-EM; the resolution limits of *in situ* approaches; or just the way proteins are (heterologously) expressed, purified and, in the case of membrane proteins, in what type of membrane mimetic they are being studied.

In summary, to gain a thorough understanding of ABC transporters, which have kept surprising us for decades and will without doubt continue to do so, we will need all of these approaches and more. Doing science is difficult enough, let us then take a lesson from the individual domains of our favourite (ABC) proteins and make sure we work together in harmony.

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