Structure of Multidrug-Resistance Proteins of the ATP-Binding Cassette (ABC) Superfamily

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Abstract: Multidrug resistance of tumors, characterized by resistance against a variety of chemically unrelated anticancer agents, can be caused by overexpression of ATP-binding cassette (ABC) proteins, such as P-glycoprotein and MRP1. These multidrug-resistance proteins are plasma-membrane proteins that actively extrude chemotherapeutic agents from the cell interior, decreasing drug accumulation and thus, allowing the cells to survive in the presence of toxic levels of anticancer agents. ABC proteins contain multispanning transmembrane domains and nucleotide-binding domains (NBDs). The NBDs are responsible for the ATP binding/hydrolysis that drives drug transport, and their structure is conserved independently of the degree of primary-sequence homology. The transmembrane domains contain the drug-binding sites that are likely located in a flexible internal chamber that is sufficiently large to accommodate different drugs. It has been recently proposed that dimerization of the NBDs induced by ATP binding is a key step for the coupling of ATP hydrolysis to substrate transport. The power stroke for substrate transport can be the formation or the dissociation of the dimers. Since the NBDs and TMDs are tightly associated, association/dissociation of the NBDs may control the "gate" of the translocation pathway, formed by intracellular loops. In the case of P-glycoprotein it seems that the power stroke for transport is ATP binding (and therefore NBD dimerization), and not hydrolysis, because the major conformational and functional changes seem to occur at this step.

Key Words: P-glycoprotein, MDR1, MRP1, ABC proteins, ATP-binding cassette, nucleotide-binding domain, chemotherapy, anticancer agents.

INTRODUCTION

Multidrug-resistance proteins that belong to the ATP-binding cassette (ABC) superfamily have been studied extensively during the last two decades. Important recent advances have been critical to our current understanding on how these membrane proteins transport a variety of chemically-dissimilar substrates across the cell membranes. However, we still do not know the precise molecular mechanism of transport, largely because structural data are very limited. Recent detailed biochemical studies and the high-resolution structures of nucleotide-binding domains and complete ABC proteins serve as bases for our current structural models of substrate transport by multidrug-resistance proteins. In this review, I summarize these recent key findings and how they can relate to the transport of anticancer agents by mammalian multidrug-resistance proteins of the ABC superfamily. Among the human multidrug-resistance proteins, the focus of the review will be P-glycoprotein (MDR1, ABCB1), and to a lesser degree the multidrug-resistance protein 1 (MRP1, ABCC1), because there is currently no structural or detailed biochemical/biophysical information on other human multidrug-resistance ABC proteins, such as BCRP (ABCG2).

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MECHANISM OF MULTIDRUG-RESISTANCE

Cancer cells are frequently resistant or develop resistance to anticancer agents during treatment. One form of drug resistance is observed against a variety of chemically unrelated agents and is known as multidrug resistance, a phenomenon that can be caused by overexpression of ABC proteins, such as P-glycoprotein and MRP1 [1, 2]. These multidrug-resistance proteins are plasma-membrane proteins that actively extrude anticancer agents from the cell interior, decreasing drug accumulation and thus, allowing the multidrug-resistant cells to survive in the presence of toxic levels of chemotherapeutic agents [1, 2]. There is evidence for the involvement of P-glycoprotein and MRP1 in the multidrug resistance of some forms of cancer, including solid tumors (e.g. breast and ovarian cancer, sarcoma and lung cancer) and hematological malignancies (e.g. acute myeloid leukemia) [1, 3-7].

Many ABC proteins display high substrate specificity, but a few of them, the multidrug-resistance proteins, are poly-specific and mediate ATP-dependent efflux of a variety of chemically dissimilar substrates. Most P-glycoprotein substrates are hydrophobic organic compounds of relatively low-molecular weight that are positively charged at normal pH [see 8-10]. MRP1 transports glutathione, glucuronate and sulfate conjugates, which include conjugates of anticancer agents and cytokines [11-16]. Other compounds are likely cotransported with reduced glutathione and/or other anions [see 16].

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Although P-glycoprotein has a number of physiological functions, its main activity seems to be the protection of the body and specific cell types from toxic agents. This is clearly suggested by its preferential locations (apical membrane of epithelial cells of the intestine and renal proximal tubule, canalicular membrane of the hepatocytes and endothelial face of the blood-brain barrier) and studies in knockout mice [see 17]. MRP1 is ubiquitously expressed [see 16] and, in addition to its role in resistance to chemotherapeutic agents, it may participate in the regulation of cell redox potential (*via* glutathione transport), ion transport (*via* ion channel regulation) and inflammatory responses (*via* transport of prostaglandins, leukotrienes and cytokines), as well as the transport of endo- and xenobiotics, and the distribution of phospholipids in membranes [14-16, 18].

GENERAL STRUCTURAL ASPECTS OF P-GLYCOPROTEIN AND MRP1

Fig. (**1**) shows the topological structure of P-glycoprotein and MRP1. The basic ABC core structure, common to both proteins, consists of a nucleotide-binding domain (NBD1) located between two transmembrane domains (TMD_1 and TMD_2), and another nucleotide-binding domain (NBD2) that follows TMD_2. MRP1 contains an additional transmembrane domain (TMD_0) linked to the core ABC domain by a linker sequence (L_o). Both proteins are glycosylated, but drug transport occurs in the absence of glycosylation, though glycosylation may play a regulatory role [16, 19, 20].

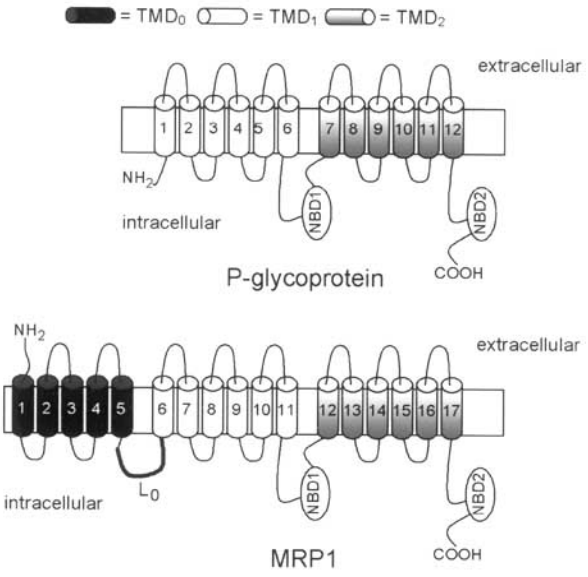

Fig. (1). Domain structure of P-glycoprotein and MRP1. Schematic representation of P-glycoprotein and MRP1. Putative transmembrane α-helices are shown as cylinders arranged in multispanning transmembrane domains (TMDs). The N- and C-terminal nucleotide-binding domains (NBD1 and NBD2) are also depicted. L_0 refers to the MRP1 linker sequence that is required for drug transport.

P-glycoprotein belongs to the ABCB subfamily of ABC proteins [21], which also includes the transporter associated with antigen processing, a heterodimer formed by TAP1 (ABCB2) and TAP2 (ABCB3). MRP1 belongs to the ABCC subfamily [1, 14, 16, 21] that also includes MRP1-MRP7, the cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7) and the sulfonylurea receptor SUR (ABCC8). Compared to other ABC proteins, ABCC proteins have a deletion of a 13 amino-acid stretch in NBD1 (most ABCC proteins) [see 16 and 22], and contain a third transmembrane domain (MSD_0), N-terminal to the basic ABC core structure (see Fig. (**1**), absent in MRP4, MRP5 and CFTR) [see 22 and 23]. The significance of the conserved NBD1 deletion is unclear, but insertion of the equivalent P-glycoprotein residues results in the expression of an inactive MRP1 [24]. L_0, but not TMD_0, is essential for drug transport [25, 26], but the nature of its involvement and the role of TMD_0 are unknown.

ABC PROTEINS

ABC proteins comprise one of the largest protein families (see http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html for human ABC transporter genes). Understanding how ABC proteins transport substrates is of high medical importance because ABC proteins are responsible for some forms of microbial resistance and multidrug-resistance of some forms of cancers [1, 2, 7, 27, 28]. ABC proteins are also the receptors for pharmacological agents (e.g. SUR for oral hypoglycemic agents used to treat type 2 diabetes) [29], and mutations of ABC genes cause genetic diseases (e.g. cystic fibrosis) [30]. Most ABC proteins are pumps that transport substrates across membranes (SUR and CFTR may be exceptions), but they also seem to have an independent regulatory function. For instance, CFTR is a Cl^- channel that also regulates other ion channels [31], and phosphorylation of P-glycoprotein also regulates Cl^- channels [32, 33].

ROLE OF THE NBDS

The overall sequence identity among ABC proteins is low, especially in the TMDs. The presence of more conserved NBDs is consistent with the notion that the varied functions of the proteins (e.g. ion channels, lipid transporters, peptide transporters) depend on the divergent TMDs; the common feature of nucleotide binding and hydrolysis, however, require the structurally conserved NBDs. Many ABC proteins from lower organisms contain 2 identical NBDs, and NBD1 and NBD2 of many other ABC proteins, including P-glycoprotein, have very similar sequences and functional properties [34-37]. In contrast, the two NBDs of other ABC proteins have very different functional roles [30, 38-41]. Proteins of this kind include those of the MRP subfamily (ABCC).

Both NBDs are required for proper function of mammalian ABC proteins, and a mandatory positive catalytic cooperativity between the NBDs occurs in P-glycoprotein [34-37]. In this protein, hydrolysis occurs at only one NBD per hydrolysis cycle, NBD1 and NBD2 are equivalent, and ATP binds randomly to either NBD [see 35 and 37]. Although there are some asymmetries between the two NBDs of P-glycoprotein, both behave fairly similarly from a functional point of view [34-37, 42, 43]. The current working hypothesis for CFTR channel gating also proposes cooperativity between NBD1 and NBD2. The simplest interpretation of single-channel analysis studies using nucleotide analogs and

mutations at NBD1 and/or NBD2 is that the main effects of ATP hydrolysis by NBD1 and NBD2 are to "open" and "close" the channel, respectively [30, 35, 44]. However, recent data point towards a more complex picture that involves a more stable nucleotide interaction and a slower rate of hydrolysis at NBD1, compared with NBD2 [30, 41, 45]. The NBDs of SUR also have distinct functions [38-40]. It has been proposed that SUR ATPase activity resides at NBD2, while NBD1 binds, but does not significantly hydrolyze ATP [40].

A model for the MRP1 catalytic cycle has been proposed based on recent findings [24, 46-50]. It is thought that binding of transport substrates induces a conformational change in NBD2 that increases its rate of ATP hydrolysis, which is coupled to substrate transport. Phosphate is released from NBD2 before ADP, and a signal is transmitted from the ADP-bound NBD2 that causes a conformational change of NBD1 that increases its affinity for ATP. The γ-phosphate of ATP molecule bound to NBD1 is transferred to MRP1 (MRP1 phosphorylation) and the ADP is released. The phosphate is then transferred to the ADP trapped in NBD2, leaving NBD1 empty and NBD2 bound to ATP, ready to start a new catalytic cycle. Although it was thought possible that NBD2 can perform hydrolysis coupled to transport in the absence of a functional NBD1 [24, 48], recent results contradict this idea [51]. However, it is evident that hydrolysis of ATP by NBD1 is much slower than that by NBD2 [24, 47-49].

In summary, the functional role of each of the NBDs in P-glycoprotein and MRP1 is quite different. In P-glycoprotein ATP hydrolysis at NBD1 and NBD2 seems to be equally important, while most ATP hydrolysis by MRP1 takes place at NBD2, with a role of NBD1 that is mostly regulatory of NBD2. The structural correlate of the differential role of the NBDs in P-glycoprotein and MRP1 is unknown.

STRUCTURE OF THE NBD

The structure of the NBDs is conserved among ABC proteins, independently of the degree of primary-sequence homology [52-62]. A model of the structure of HisP, the ATP-binding subunit of a histidine permease, is shown in Fig. (**2A**) [52, see 63 and 64 for reviews and 61 for a detailed discussion]. Each NBD is formed by two arms. One (Arm I in Fig. (**2A**)) contains the basic core domain, homologous to the F_1-ATPase (red α helices and β sheets), with the conserved motifs A and B (see Fig. (**2B**)). An antiparallel β-sheet subdomain that interacts with the sugar and base moieties of the nucleotide is also contained in this arm (ABCβ, yellow β sheets). The basic aspects of binding of the α and β nucleotide phosphates and Mg^{2+} are conserved between ABC proteins and the F_1-ATPase [61, 64, 65]. They share a "Rossman fold" consisting of a central core of β sheets surrounded by α helices [61, 64, 65]. Motifs A and B are more than 90 amino acids apart in the primary sequence of ABC proteins (Fig. **2B**), but are close to each other in the 3-D structure. Motif A (also called P-loop) is a pyrophosphate-binding site that binds the β and γ phosphates of nucleotide triphosphates [66, 67]. It is located between a β sheet and an α helix (red β1 and α1 in Fig. (**2B**)). Most hydrogen bonds are formed between the main-chain nitrogens and the phosphates. Therefore, the residue side chains can vary, consistent with the poor conservation of the primary sequence of the central region of motif A (Gly-**X**$_4$-Gly-Lys-Thr/Ser). The conserved Lys contributes most of the binding energy of the pyrophosphate/motif A interaction. Motif B is in a highly hydrophobic β sheet that ends in an acidic residue that interacts with Mg^{2+}. The acidic residue is not the catalytic carboxylate, but it is necessary for completion of the hydrolytic cycle [51, 68].

The other arm of the NBDs (arm II in Fig. (**2A**)) contains a structurally conserved α-helical subdomain (ABCα, green α helices, Fig. (**2**)). This subdomain contains 3 α helices and motif C (signature sequence), the most conserved sequence among ABC proteins. It is likely that this subdomain participates in nucleotide binding (see **Association between NBDs**) and also interacts with the TMDs [57, 62]. In the hinge region between the arms, the so-called Gly (approximately equivalent to the γ-phosphate linker labeled in Fig. (**2B**), purple loop in Fig. (**2A**)) and His loops interact through conserved Gly and His residues with Mg^{2+} and are involved in the presentation of the hydrolytic water [61].

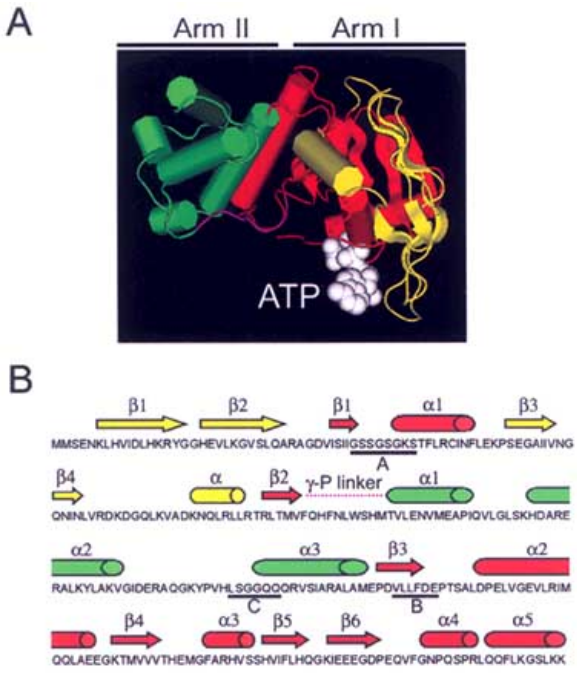

Fig. (2). Structure of the nucleotide-binding domains. A. Structure of the ATP-binding subunit of the *Salmonella thyphimurium* histidine permease, HisP. Model NBD structure where α helices and β sheets are shown as cylinders and flat arrows, respectively. B. Primary and secondary structure of HisP. Cylinders and arrows depict α helices and β sheets, respectively. The conserved motifs A, B and C are underlined, and the γ-phosphate linker (Gly loop) is marked by a purple dot line. See text for details and color labeling of the subdomains.

The molecular mechanism of nucleotide hydrolysis by ABC proteins has not been elucidated, but is likely that involves approximation of ATP and Mg^{2+} and base activation of the attacking water. The activating base is likely a

nucleophilic side chain that donates an electron pair to a water molecule, increasing its nucleophilicity. This "activated" water then attacks the bond between the β- and γ-phosphates.

ASSOCIATION BETWEEN NBDS

Proper function of ABC proteins requires two “normal” NBDs [34-36, 42, 43, 47-49]. Nucleotide binding to the NBD monomers elicits conformational changes *via* an induced-fit [59] mechanism, which do not seem to be responsible for the power stroke that couples substrate transport and ATP hydrolysis [59, 61, 69]. Recent evidence suggests that the two NBDs interact physically, and it has been proposed that this interaction provides the power stroke during the transport cycle [69].

Several structural arrangements in the dimers identified or proposed for the crystallized NBDs are quite different (see Fig. (**3**)) [see 64]. A “back-to-back” arrangement was proposed for the HisP dimers [52], with the arm II structures of each monomer forming a common TMD-interacting surface, and motifs A and C of each monomer facing away from the corresponding motifs of the other monomer. As a consequence, the NBD- bound ATPs are ca. 40 Å apart and motif C does not form part of the nucleotide-binding site.

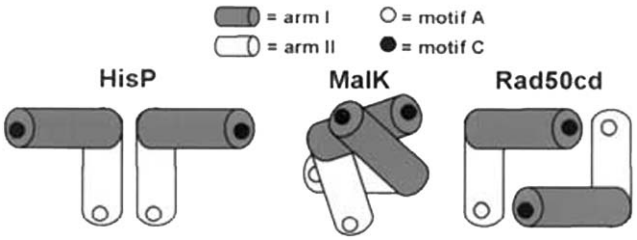

Fig. (3). Proposed spatial arrangements of NBDs. Schematic representation of the NBD dimers originally proposed for HisP, MalK and Rad50CD. Modified from [64].

For MalK, the ATP-binding subunit of the bacterial maltose permease, a slightly asymmetric interlocking association was proposed [53]. The motifs A of each NBD are in front of each other, but about ca. 40 Å apart, while the motifs C are only ca. 18 Å apart. The most interesting arrangement is that obtained from the analysis of crystals of Rad50, a soluble protein involved in DNA repair. This protein has catalytic domains related to the NBDs of ABC proteins. ATP binding induces the formation of dimers of the N- and C-terminal catalytic domains of Rad50 (Rad50 catalytic domain, Rad50CD) [54]. In the Rad50CD dimer, obtained in the presence of a non-hydrolyzable ATP analog, each of the two ATP binding sites is formed by residues in motifs A and B from one monomer and residues from motif C of the other monomer [54]. Motifs A and B interact with the ATP pyrophosphate moiety and motif C contributes additional interactions with the phosphate, adenine and ribose [54]. The structure of the complete ABC protein Btu (vitamin B_{12} importer) resembles that of Rad50CD, and also suggests that there is some separation of the NBDs in the absence of nucleotide [62]. Studies of archaebacteria NBDs support a Rad50-like arrangement because ATP binding induces dimerization of NBDs, forming a nucleotide sandwich dimer [69, 70]. Recent experiments indicate that during ATP hydrolysis motif A of one NBD and motif C of the other NBD are close to each other in the maltose transporter complex [71]. The Rad50CD model requires two NBDs for ATP binding and hydrolysis and therefore, explains the cooperativity between the two NBDs in ATP hydrolysis by ABC proteins. A representation of the dimer structure of the *Methanococcus jannaschii* NBD MJ0796 is shown in Fig. (**4**). The crystal structure was obtained using the E171Q mutant that is hydrolytically inactive and allows for the stable formation of an ATP-sandwich dimer [69]. The structure shows that each ATP (white, sphere atomic representation) is sandwiched between the core domain of one monomer (red or magenta, containing motifs A and B) and the α-helical subdomain of the other monomer (green or lime, containing motif C).

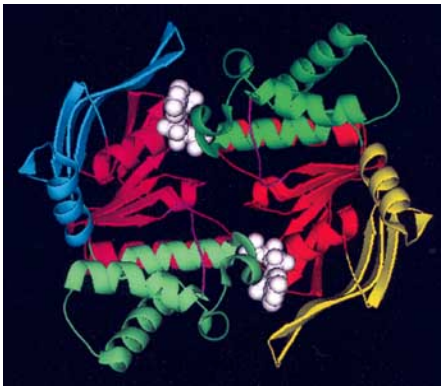

Fig. (4). Dimer structure of the nucleotide-binding domains. The dimer structure of the MJ0796 E171Q mutant of the *Methanococcus jannaschii* NBD is presented with α helices and β sheets shown as cylinders and flat arrows, respectively. The subdomain color coding for the monomer on the right is the same as that in Fig. (**2**): red, green and yellow for the ATP-binding core, α-helical and antiparallel β-sheet subdomains, respectively. The corresponding domains of the monomer on the left are depicted in magenta, lime and cyan, respectively. The γ-phosphate linker is shown in purple and the ATP molecules in white.

The characteristics of the interaction between NBDs in functional ABC proteins with the complete core domain structure (P-glycoprotein and MRP1) are unknown. However, cysteine crosslinking experiments on P-glycoprotein suggest a Rad50CD-like NBD arrangement, at least at some stages during the ATP binding/hydrolysis cycle [72]. In apparent contrast with the Rad50CD-like model and most of the available data, recent mutagenesis studies of SUR suggest that motif C is not directly involved in ATP binding [73]. Therefore, additional experiments using functional ABC proteins are needed to resolve the important issue of the nature of the physical interactions of the NBDs during the transport cycle in functional proteins.

It is possible that isolated ABC NBDs, such as HisP [52] and MalK [53], and the complete ABC protein MsbA (it

shows the 2 NBDs far away from each other [57]) actually crystallized as monomers, and the proposed dimeric structures do not reflect the native arrangements (i.e. intermolecular forces present during crystal formation produced contacts between the monomers that are not the biologically-relevant dimer associations).

In summary, most of the data available support a Rad50CD-like NBD dimer structure. Crystallization artifacts can explain some of the conflicting results, but different NBD arrangements in different ABC proteins and/or the dynamics of the NBD associations during the transport cycle can also explain some of the discrepancies.

HIGH-RESOLUTION STRUCTURE OF ABC PROTEINS

Although no high-resolution structure of an ABC multidrug-resistance protein has been solved, two structures of *E. coli* ABC proteins became available recently; those of the lipid A flippase MsbA [57] and the vitamin B_{12} transporter Btu [62]. These two transporters perform very different functions and their structure is significantly dissimilar in key aspects.

MsbA is a flippase that transports lipid A from the inner to the outer leaflet of the *E. coli* plasma membrane. Lipid A is a major component of the outer membrane that is essential for cell viability. The analysis of the MsbA structure is of interest to gain insight into the multidrug-resistance proteins because it is one of the bacterial ABC transport proteins that is phylogenetically closer to P-glycoprotein. P-glycoprotein and the close functional homolog LmrA (*Lactococcus lactis* multidrug transporter) are all believed to be flippases [74-76], suggesting common ancestry. The crystal structure of MsbA has been solved to 4.5 Å [57]. The protein has a domain structure that resembles a "half P-glycoprotein" and forms homodimers (see Fig. (**5**)). Each monomer has one TMD (one is shown in purple and the one from the other monomer in green), one NBD (shown in red) and one additional domain linking the previous two (intracellular domain, ICD, shown in yellow). The homodimer length is approximately 120 Å, with ca. 52 Å corresponding to the putative membrane-spanning region. It has 12 transmembrane α-helices (6 *per* monomer), all tilted 30-40^{o} with respect to the membrane normal, that form a cone-shaped chamber. This chamber is opened to the intracellular side, with an open wide base of ca. 45 Å. The vertex of the chamber seems closed on the extracellular side. The dimer interface is formed mostly by residues located in the periplasmic region of transmembrane helices 2 and 5 (TM2 and TM5), and two large gaps in the α-helices-delimited chamber are clearly evident between the TMDs of each monomer. Fig. (**5**) shows a view into the chamber from one such gap. It seems possible that the chamber is partially filled with lipids and that the gaps allow the access of lipid A from the inner leaflet of the membrane into the chamber. There is a cluster of positively charged residues (4 Arg and 2 Lys) near the vertex of the chamber, while the remaining part of this area is a significantly more hydrophobic environment.

As mentioned under **Association of NBDs**, the NBDs are far away from each other, at least in the absence of nucleotides [57]. The structured regions in ICD are mostly α-helical. Particular α-helices in contact with the NBD and just preceding it are highly conserved in multidrug-resistance ABC proteins, and may be involved in the transmission of signals between the TMDs and the NBDs [57].

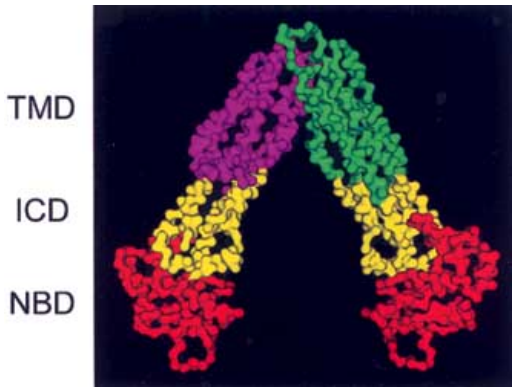

Fig. (5). Structure of the *E. coli* lipid A flippase MsbA. The protein is shown viewed from the side and looking from the lipid bilayer into the chamber. The transmembrane domains (TMD) of each monomer are depicted in green and purple. The intracellular (ICD) and nucleotide-binding domains (NBD) are shown in yellow and red, respectively. The location of the NBDs is intracellular.

It is possible that the MsbA dimer structure shown in Fig. (**5**) is artifactual, and it has been proposed that a rotation of the monomers to form a chamber open to the periplasmic space allows for an adequate fit into the low-resolution structure of P-glycoprotein (see **Low-resolution structures of ABC multidrug-resistance proteins** below).

A model for lipid A transport by MsbA has been proposed [57]. In this model binding of lipid A to MsbA stimulates nucleotide binding *via* a conformational change in the TMDs transmitted to the NBDs by the ICD. Nucleotide binding produces an approach of the NBDs that together with movements in TM2, TM5 and TM6 closes the chamber. The cluster of positive charges on the cytoplasmic side of the chamber creates an energetically unfavorable environment for the hydrophobic lipid A, which flips into the more hydrophobic environment in the outer leaflet portion of the chamber, forming hydrophobic interactions. The flipping of the lipid or the closing of the chamber can trigger ATP hydrolysis by the NBDs, rearranging the chamber to promote the movement of the lipid to the outer leaflet of the membrane, and eventually resetting the protein for a new transport cycle.

The structure of the *E. coli* vitamin B_{12} importer BtuCD has been recently solved to 3.2 Å resolution by X-ray diffraction analysis of 3-D crystals (Fig. **6**) [62]. The functional BtuCD is a tetramer formed by two identical TMDs (BtuC, one is shown in purple and the other one in green) and two identical NBDs (BtuD, one shown in red and the other one in yellow). The complex is approximately 90 Å tall, 60 Å wide and 30 Å thick when viewed from the front face. Its shape resembles that of an inverted portal with a large water-filled opening, below the TMDs, between the 4 subunits (Fig. **6**). As described under **Association of NBDs**, the arrangement of the NBDs resembles that of Rad50CD and MJ0796 (see Fig. (**3**) and Fig. (**4**)) [54], and is very

different from that in MsbA (Fig. **5**). In addition, in BtuCD the NBDs are located just below the TMDs, because the protein does not have a large additional domain between the TMDs and NBDs (see yellow ICD in Fig. (**5**)).

Each TMD contains 10 transmembrane α-helices, packed together in a very different way than in MsbA. A pseudo two-fold rotation axis relates two groups of 4 consecutive α-helices (TM2 to TM5 and TM7 to TM10). Because of the structural resemblance between the two helix groups, it is possible that the groups represent basic building blocks in ABC proteins. The interface between the TMDs is formed by antiparallel packing of TM5 of one TMD and TM10 of the other. The chamber between these four helices is open to the periplasmic space and is large enough to accommodate vitamin B_{12}. The chamber interior is lined by hydrophobic residues from TM5 and TM10, as well as the regions preceding TM3 and TM8. The cytoplasmic turn between TM4 and TM5 forms a gate (Fig. (**6**), residues shown in white) that closes the putative translocation pathway on the cytoplasmic side. The loop between TM6 and TM7 forms two short α-helices connected by a sharp turn (blue in Fig. (**6**)). It is known as L loop because of its shape, and forms extensive contacts with the NBDs. It may represent a general interface between the TMDs and NBDs based on the importance of the homologous sequence in a number of ABC proteins, including P-glycoprotein, as demonstrated by mutagenesis studies [77]. The corresponding region in MsbA also contacts the NBD [57]. The L loop is likely involved in the transmission of signals between TMDs and NBDs (analogous to the proposed function of the ICD in MsbA).

The association between BtuC and the periplasmic vitamin B_{12} binding protein BtuF (not shown) is likely to occur through salt bridges between two Glu in BtuF (ca. 46 Å apart) and 2 sets of 3 Arg residues (ca. 48 Å apart from each other) [78]. It has been proposed that docking of the substrate-bound periplasmic protein to BtuC produces a conformational change in the TMDs that is transmitted to the NBDs *via* the L loop [62, 78]. This results in ATP binding that is coupled to the opening of the gate that closes the cytoplasmic side of the translocation pathway. In the proposed model, swinging of the NBDs toward each other applies a force to the diametrically opposed contact region of the L loop that is transmitted to the TMDs, opening the gate [see 62]. Vitamin B_{12} will move into the cytoplasm, passing through the large hydrophilic area just below the TMDs. Transport will be by diffusion or peristaltic forces exerted by the TMDs. Resetting of the transporting will occur following the release of ADP and phosphate. This model takes into account the observation that the NBDs are close together even in the absence of ATP [62]. However, it is possible that hydrolysis leads to separation of the NBDs, opening the gate. The alternative that the gate is always closed and vitamin B_{12} moves, through a gap created in the chamber, into the inner leaflet of the membrane, and from there to the cytoplasm, cannot be ruled out either.

The potential for extrapolation of important basic aspects of the proposed mechanisms of substrate transport by MsbA and BtuCD to the transport of chemotherapeutic agents by P-glycoprotein and MRP1 is still unclear. The low degree of homology in the primary sequence of the transmembrane domain segments, the very different functions among ABC proteins, and major differences in helix packing between MsbA and BtuCD point to the possibility of varied basic arrangements and mechanisms of transport among ABC proteins. Unfortunately, there is essentially no biochemical information on MsbA and Btu to help in the analysis of the structures, and the generation of transport models. It is expected that new high-resolution crystal structures and detailed biochemical/biophysical information will help with the building of more specific models of transport by ABC proteins.

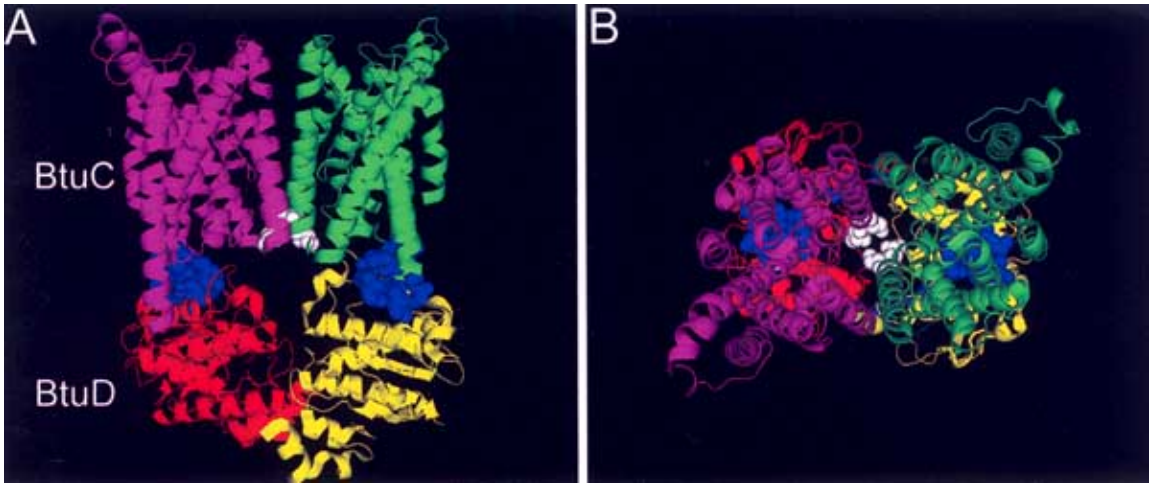

Fig. (6). Structure of the vitamin B_{12} importer BtuCD. Each of the four subunits that form the functional unit (together with the periplasmic-binding protein BtuF, not shown) is colored coded. The two identical subunits that contain the transmembrane domains (BtuC) are shown in purple and green, and the two identical subunits that contain the NBDs (BtuD) are shown in red and yellow. The NBDs are intracellular. The backbone of the conserved L-loop that makes contacts with the NBDs, and is probably involved in transmission of signals between the TMDs and NBDs, is shown in blue. The space filled by Thr^{142} and Ser^{143}, that form the putative gate of the translocation pathway, is shown in white. A side view **(A)** and a view from the periplasmic space into the chamber **(B)** are shown.

LOW-RESOLUTION STRUCTURE OF ABC MULTIDRUG-RESISTANCE PROTEINS

Although high-resolution structures of multidrug-resistance proteins of the ABC superfamily are not available, important information has been obtained from transmission electron microscopy of both reconstituted and detergent-solubilized P-glycoprotein, as well as cryoelectron microscopy of 2-D crystals [79, 80]. The studies with fully active P-glycoprotein reconstituted in a lipid bilayer are important for validation of higher-resolution structures from 2-D and 3-D crystals, where the function of the protein cannot be tested. In the low-resolution studies available to date [79-81], P-glycoprotein shows as an approximately 110 Å-long particle, 80 Å-wide, with a central low-density chamber that opens wide (ca. 50 Å) to the extracellular side (similar to BtuCD, Fig. (**6**)) [62]. A large gap in the TMDs lining the chamber, which may be analogous to the gaps in the MsbA chamber [57], is particularly evident upon ATP binding [81]. In the absence of ATP the central pore seems closed at the bottom, but in the ATP-bound protein (bound to the non-hydrolyzable ATP analog PNP-AMP) the closure is not evident [81]. Calculations of relative distances between the NBDs and between the NBDs and the membrane, based on Förster resonance energy transfer using fluorescent labels, generally agree with the determined P-glycoprotein structure [see 82]. A low-resolution structure of MRP1 shows a probable dimer, in which MRP1 has similar size and shape than P-glycoprotein, with a protein electron density on the outer side of the ring that may represent the additional TMD (TMD_0) [83].

As a note, it is important to mention that rotation of MsbA monomers (homology modeling its NBDs based on the MJ0796 dimer in Fig. (**4**)) creates an extracellularly opened chamber compatible with the low-resolution structure of P-glycoprotein [81, 84]. However, this does not solve all controversies on the basic structural aspects of multidrug-resistance proteins because the chamber of functional LmrA is opened towards the intracellular solution in the absence of nucleotides and drugs [85], as in the original MsbA structure [57].

Some of the conformational changes that occur in P-glycoprotein during the transport cycle, identified from biochemical studies, have been directly observed by the changes in P-glycoprotein structure in the absence of ligands, locked in the ATP-bound state (non-hydrolyzable ATP analog AMP-PNP) or in a post-hydrolytic state (bound to vanadate and ADP) [80, 81]. The most surprising observation is that the largest change occurs in response to ATP binding [80, 81]. The combination of the structural and functional data on P-glycoprotein lead to the controversial conclusion that the power stroke for drug transport is ATP binding, and that hydrolysis serves to reset the transporter for a new transport cycle [80]. Nucleotide binding produces a large change in the overall shape of the protein as a result of a major reorganization of the TMDs throughout the entire depth of the membrane, which coincides with a decrease in drug-binding affinity [80, 81]. An additional conformational change takes place post hydrolysis, where the affinity for drugs is still low [86, 87]. This may facilitate the release of the drug to the extracellular solution or may be an intermediary step towards the resetting of the transporter to the high drug-affinity state. Consistent with an increased solvent exposure of the drug-binding pocket, spectroscopy studies, especially those using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), show that there is a sizable change in residue accessibility to the solvent in response to ATP hydrolysis [see 82].

Unfortunately, the resolution of the P-glycoprotein structures is insufficient to define secondary structure elements. However, biochemical crosslinking data consistent with the low-resolution structure of P-glycoprotein (i.e. chamber open to the extracellular side with maximum width of 50 Å) [88] showed that the drug-binding site is formed by α-helices 4-6 and 10-12 [89, 90] and that the chamber opening in the middle is of ca. 10-25 Å [88]. A left-handed coiled-coil arrangement of TM6 and TM12 with the helices close to each other across their entire length seems possible [91]. Interestingly, crosslinking between two residues, one in TM6 and the other in TM12 was enhanced with ATP hydrolysis, but not ATP binding, and several substrates blocked crosslinking between pairs of residues in TM6 and TM12 [91]. These data constitute evidence for helix rotation with ATP hydrolysis. There is also evidence of significant and varying changes in crosslinking patterns by binding of drugs, such as progesterone, cyclosporine and colchicines [92]. This supports the notion that substrate binding produces rotation and/or lateral movement of helices that can accommodate varying substrates by an induced-fit mechanism. This induced-fit mechanism is consistent with the detailed data available for the transcriptional regulator QacR [93-95].

Recent observations suggest that the structural changes during the drug transport/ATP hydrolysis cycle in MRP1 are different from those in P-glycoprotein [96]. Because of the very limited structural information available, the MRP1 cycle is not discussed here.

THE DRUG-BINDING POCKET OF MULTIDRUG-RESISTANCE PROTEINS

The idea that there is a hydrophobic drug-binding pocket in multidrug-resistance proteins, where hydrophobic substrates bind to the protein without specific interactions can be ruled out based on large differences in affinity for similar compounds (the proteins are poly-specific rather than non-specific) [see 8-10, 16]. Extensive studies on P-glycoprotein have provided only limited information on the substrates requirements [8-10]. Hydrophobicity due to the presence of planar aromatic groups is important. A basic nitrogen atom is frequently encountered in good substrates, and a tertiary amine seems associated with high-affinity interaction with P-glycoprotein. Hydrogen bonds play major roles in the protein-drug interaction, with relevant electron donor groups in the drugs that are spatially separated by 2.5 or 4.6 A. Mutations in P-glycoprotein and MRP1 helix residues that can form hydrogen bonds support their role in the drug-protein interaction [97, 98].

Many multidrug-resistance proteins can be found outside the ABC superfamily [99, 100]. The other families that include multidrug-resistance proteins are the major facilitator superfamily, (MFS), the small multidrug-resistance family

(SMR), the resistance-nodulation-cell division family (RND) and the multidrug and toxic compounds extrusion family (MATE). Interestingly, there are several common substrates for all the multidrug-resistance proteins (e.g. tetraphenylphosphonium, rhodamine 6G and ethidium bromide), which suggest common structural aspects of the multidrug binding sites [see 10]. However, the similarities are not clear because the helix arrangement and the presence of a central pore are not common features. For instance, the membrane spanning helices of the *E. coli* multidrug-resistance protein EmrE (SMR family) are tightly packed in an asymmetric tight bundle, without a central pore [101].

Recent high-resolution structures of soluble proteins that interact with chemically and structurally dissimilar compounds have provided important insight on the nature of multidrug-resistance binding pockets [94, 95, 102-104]. For example, transcriptional regulators of bacterial multidrug-resistance proteins (e.g. BmrR and QacR) bind many of the same cationic substrates transported by the proteins whose expression they regulate. Binding of the drugs to the transcriptional regulators is characterized by their penetration into a hydrophobic binding pocket, where they make van der Walls and stacking interactions. The cationic drugs also make an electrostatic interaction with buried anionic residues. The presence of charge residues in the transmembrane helices is also important for interaction with substrates in SMR and MFS multidrug-resistance proteins [e.g. 105, 106]. A Glu in TM14 of MRP1 is also important for transport of the cationic anthracyclines, but not anionic substrates, such as leukotriene C4 [107]. P-glycoprotein does not have TM charged residues, but cations can bind to the π phase of aromatic side chains. This electrostatic interaction in the hydrophobic environment can be strong. In support of this notion, mutations in residues with aromatic side chains affect P-glycoprotein substrate specificity and/or affinity, and hydrogen-bonding side chains are often clustered on one side of the helices [89, 90, 108].

The binding pocket of the soluble transcriptional regulators is flexible and large enough to accommodate different drugs and it is possible for two different drugs to be accommodated in the pocket with very little overlapping [93, 104]. In a promiscuous xenobiotic receptor with a binding pocket structurally similar to that of the multidrug-resistance transcriptional regulators, a single drug can also interact with pocket residues in different orientations [108]. Parts of the binding pocket are flexible and change conformation upon binding to specific drugs [93]. An induced-fit mechanism of this kind seems likely for the interaction of P-glycoprotein with some of its substrates [92]. It is well established that ABC multidrug transporters have more than one drug interaction site [see 10], and the structure of the binding pocket of the transcriptional regulators suggests that the binding sites are located in a single flexible binding region, as opposed to different regions.

MOLECULAR MECHANISM OF TRANSPORT

Dimerization of the NBDs of ABC proteins can explain the cooperativity between the NBDs and also provide a general mechanism of coupling between ATP hydrolysis and substrate transport. A speculative model for the catalytic cycle of P-glycoprotein can be presented based on recent biochemical/biophysical and structural data on ABC proteins and NBDs, as well as biochemical and structural studies on P-glycoprotein [see 37, 54, 57, 59, 62, 69, 70, 80, 81, 86-88, 91, 92]. The drug can access a high-affinity hydrophobic drug-binding site from the inner leaflet of the membrane or the cytosol [74, 111], and its binding to the protein produces a conformational change in the NBDs that increases their affinity for ATP [80, 110]. Binding of one ATP to each NBD monomer occurs by interaction with the core and antiparallel subdomains, producing a rotation in the α-helical subdomain that is coupled to the interaction of the γ-phosphate of ATP with the conserved Gly in the γ-phosphate linker (see Fig. (**2**) and Fig. (**4**)). ATP binding causes NBD dimerization because of the interaction of the bound ATP with motif C of the other monomer, sandwiching two ATP molecules between the NBDs (see Fig. (**4**)). Hydrolysis of one ATP produces a conformational change in the TMDs that involves movement of the helices that form the drug-binding pocket [90, 91], with a decrease in drug-binding affinity. The conformational change increases the exposure of the drug to a more hydrophilic environment in the chamber (reducing binding affinity), from where it diffuses to the extracellular solution or is expelled by peristaltic forces exerted by the TMDs. ATP hydrolysis also produces a major decrease in ATP-binding affinity at the other NBD [37, 86, 87], which may result in the release of the non-hydrolyzed ATP. The electrostatic repulsion between the ADP bound to motif A of one monomer and the phosphate bound to motif C of the other monomer destabilizes the dimer state. Phosphate is rapidly released after hydrolysis and ADP is release afterwards (it is the rate limiting step of the hydrolysis cycle) [see 37, 112]. The drug-binding site, however, remains in a low drug-binding-affinity state and hydrolysis of another ATP is required for resetting the transporter for a new drug-transport cycle [see 37, 87]. Some interesting aspects of the proposed P-glycoprotein catalytic cycle are that hydrolysis occurs at one NBD at any given time, the NBDs are recruited randomly for each hydrolysis cycle, and transport of one drug is coupled to hydrolysis of two ATP molecules [see 37]. If the P-glycoprotein catalytic cycle does include hydrolysis of 2 ATPs *per* cycle, as supported by the data and the model proposed by Ambudkar and co-workers [see 37], it is possible that two NBD association/dissociation events occur *per* transport cycle, one coupled to drug transport and the other to resetting of the transporter for a new cycle.

The power stroke for substrate transport can be the formation or the dissociation of the dimers [69, 70]. Since the NBDs and TMDs are tightly associated, association/dissociation of the NBDs may control the "gate" of the translocation pathway formed by intracellular loops. Tightening of helix packing on the cytoplasmic side could be the key step for substrate efflux (e.g. in P-glycoprotein) and the opposite could be true for substrate influx (Btu). Alternatively, the gate of the chamber on the cytoplasmic side may always be closed and the dimerization of the NBDs could produce conformational changes in the TMDs that reduce drug-binding affinity and also allow for substrate movement from (for efflux) or to (from influx) the inner leaflet of the membrane. Recent data on LmrA, however, show access of the hydrophilic chamber to the cytosolic side

in the absence of drugs and ATP. Independently of the molecular mechanism, it seems that for P-glycoprotein the power stroke for transport is ATP binding, as opposed to hydrolysis, because the major conformational and functional changes seem to occur at this step. It is possible that this is the case for ABC efflux pumps, while hydrolysis provides the power stroke for the ABC importers by opening the gate of the translocation pathway.

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ABBREVIATIONS

ABC	=	ATP-binding cassette
Btu	=	*E. coli* vitamin B_{12} transporter
CFTR	=	Cystic fibrosis transmembrane conductance regulator
HisP	=	Nucleotide-binding subunit of the histidine permease complex
ICD	=	Intracellular domain of MsbA
LmrA	=	*Lactococcus lactis* multidrug-resistance protein
MalK	=	Nucleotide-binding subunit of the maltose permease complex
MRP	=	Multidrug-resistance protein
MsbA	=	*E. coli* lipid A transporter
NBD	=	Nucleotide-binding domain
NBD1	=	N-terminus nucleotide-binding domain
NBD2	=	C-terminus nucleotide-binding domain
Rad50CD	=	Catalytic domain of the DNA-repair enzyme Rad50
SUR	=	Sulfonylurea receptor
TAP	=	Transporter associated with antigen processing
TM	=	Transmembrane helix
TMD	=	Multispanning transmembrane domain

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