
Shedding light on drug transport: structure and function of the P-glycoprotein multidrug transporter (ABCB1)

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Abstract

P-Glycoprotein (Pgp; ABCB1), a member of the ATP-binding cassette (ABC) superfamily, exports structurally diverse hydrophobic compounds from the cell, driven by ATP hydrolysis. Pgp expression has been linked to efflux of chemotherapeutic drugs in human cancers, leading to multidrug resistance (MDR). The protein also plays an important physiological role in limiting drug uptake in the gut and entry into the brain. Substrates partition into the lipid bilayer before interacting with Pgp, which has been proposed to function as a hydrophobic vacuum cleaner. Low and medium resolution structural models of Pgp suggest that the two nucleotide binding domains are closely associated to form a nucleotide sandwich dimer. Pgp is an outwardly-directed flippase for fluorescent phospholipid and glycosphingolipid derivatives, which suggests that it may also translocate drug molecules from the inner to the outer membrane leaflet. The ATPase catalytic cycle of the protein is thought to proceed via an alternating sites mechanism, although the details are not understood. The lipid bilayer plays an important role in Pgp function, and may regulate both binding and transport of drugs. This review focuses on the structure and function of Pgp, and highlights the importance of fluorescence spectroscopic techniques in exploring the molecular details of this enigmatic transporter.

Introduction

The ATP binding cassette (ABC) superfamily is one of the largest protein families distributed among all the kingdoms of life (Dassa and

Schneider 2001). They play a critical role in human health and are responsible for several important diseases (Borst and Oude Elferink 2002). These proteins are usually built from 4 modules, two transmembrane (TM) domains and two nucleotide-binding (NB) domains. In bacteria, these four modules may exist as separate subunits, while in mammals, ABC proteins often consist of a single polypeptide chain. Most ABC proteins are membrane transporters, either importing (bacteria) or exporting (mammals) their substrates, driven by the energy of ATP hydrolysis. The range of substrates transported by ABC proteins is astonishing, and includes chloride ions, amino acids, drugs, small peptides and large proteins. In recent years, significant advances have been made in determining the high resolution molecular structures of ABC proteins, which has in turn led to a better understanding of their possible mechanism of action (Jones and George 2004). However, many important details still remain to be uncovered.

P-Glycoprotein (Pgp; ABCB1, MDR1) is one of the most intensively studied ABC family members. This 170 kDa protein was first discovered in the plasma membrane of mammalian cells that had been selected for resistance to drugs (Gottesman and Ling 2006). Over a period of several years, it became clear that Pgp functions as an ATP-driven efflux pump for drugs. The protein has been implicated in the resistance of human tumour cells to multiple chemotherapeutic drugs (multidrug resistance, MDR), which is a major barrier to the successful treatment of many human cancers (Gottesman 2002).

This review is not intended to be a comprehensive examination of all the available literature on Pgp, which is extensive. Rather, it focusses on the application of various fluorescence spectroscopic approaches to study this transporter.

Pgp substrates and modulators

Pgp can interact with hundreds of structurally-diverse compounds. Transport substrates include natural products, chemotherapeutic drugs, steroids, fluorescent dyes, linear and cyclic peptides, ionophores, etc. (see Fig. 1). Most are weakly amphipathic and relatively hydrophobic; many (but not all) contain aromatic rings and a positively charged tertiary N-atom. There is little information on which, if any, of these substrates might be “physiological”, although peptides, platelet-activating factor, lipids, steroid hormones, and small cytokines are likely candidates. Direct measurement of transport has been carried out for only a few of these substrates. The majority were identi-

fied indirectly by resistance to cytotoxicity in cells overexpressing Pgp.

A second group of compounds, known as modulators (also called chemosensitizers, reversers or inhibitors), is able to reverse MDR in intact cells by blocking the drug efflux activity of Pgp (Tan et al. 2000). Most modulators appear to bind to Pgp at the substrate binding pocket, and compete with transport substrates in a complex fashion. Indeed, many modulators, including verapamil and cyclosporin A, are known to be transported by Pgp. Pgp modulators also belong to many different structural classes (see Fig. 1), and have similar molecular features to transport substrates (Wiese and Pajeva 2001). Cells are generally not resistant to killing by modulators, but a combination of MDR drugs and a modulator is highly cytotoxic.

Modulators are important clinically, since their co-administration with drugs that are Pgp transport

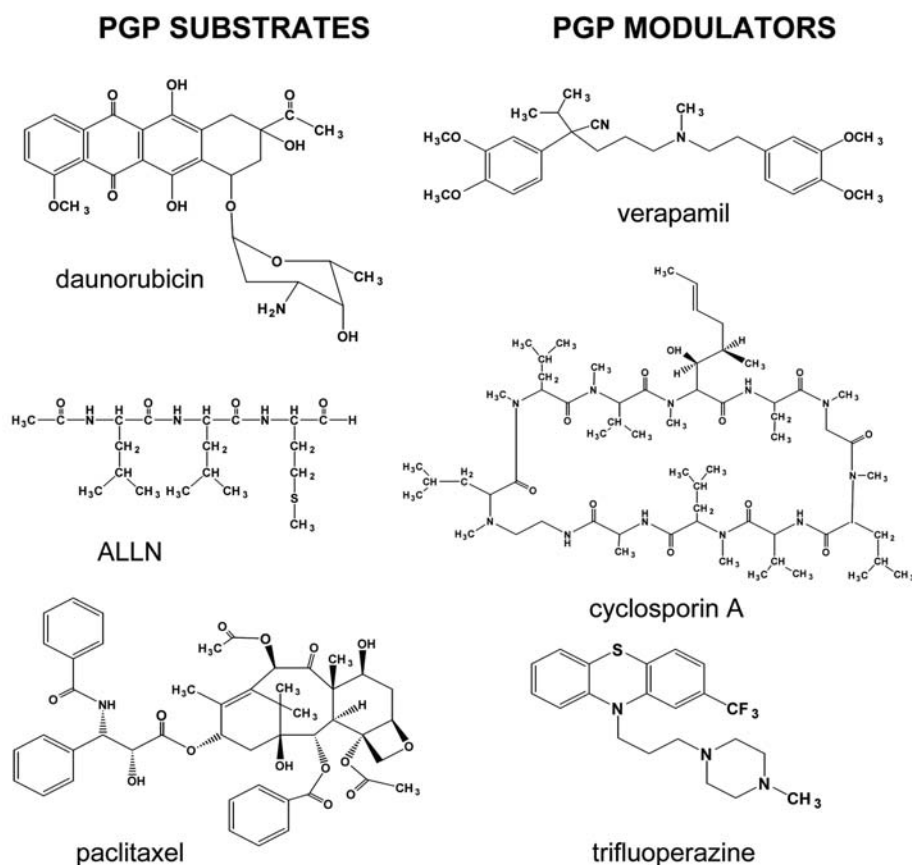


Figure 1 Structures of some Pgp substrates and modulators.

substrates has the potential to improve uptake in the gut and delivery to the brain (see below), and increase the cytotoxicity of anti-cancer drugs to tumour cells. Several promising “third-generation” modulators have progressed to clinical trials (for a review, see Modok et al. 2006). However, in general this approach has been disappointing, despite substantial evidence that treatment failure and patient survival are linked to Pgp expression in several malignancies (Polgar and Bates 2005).

Physiological role of Pgp

Pgp is now known to play a central role in the absorption and distribution of drugs in many organisms (Fromm 2003). It is expressed at the apical surface of epithelial cells lining the gastrointestinal tract, and also on the apical surface of the endothelial cells that line the brain capillaries, where it forms a major component of the blood brain barrier. Its physiological role appears to involve preventing entry of potentially toxic compounds from the gut into the blood (Zhang and Benet 2001), and protection of sensitive internal organs such as the brain from compounds that gain access to the circulation (Fromm 2004).

Transgenic knockout mice lacking Pgp are phenotypically indistinguishable from wild-type mice under normal conditions, however, they display a disrupted blood brain barrier, and are hypersensitive to drugs (Schinkel 1999). Many drugs that are used in the treatment of human disease are Pgp substrates. As well as anti-cancer drugs, these include immunosuppressive agents, HIV protease inhibitors, antibiotics, cardiac glycosides, and many more. Pgp can thus reduce the oral bioavailability of therapeutic drugs, and the targeting of such drugs to the brain tissue, limiting the efficacy of treatment.

A closely-related gene encodes a protein (ABCB4; 75% sequence similarity to Pgp) that is not a drug transporter, but an exporter of phosphatidylcholine (PC) into the bile (Ruetz and Gros 1994). This protein is believed to function as an outwardly-directed phospholipid flippase. ABCB4 is highly expressed at the bile canalicular membrane of hepatocytes.

Experimental systems for studying Pgp-mediated drug transport

Early work on MDR used mammalian cell lines selected for growth in high levels of drugs such as colchicine and vinblastine. However, simpler sub-cellular systems were soon developed to avoid the complexities of intact cells. Plasma membrane vesicles proved to be very useful, and allowed characterization of the transport process using radiolabelled drugs (for example, see Doige and Sharom 1992). Inside-out vesicles present in the preparation transport drug into the lumen when ATP and an ATP-regenerating system are added to the vesicle exterior. Purification of Pgp was necessary for further biochemical characterization of the protein. Several research groups succeeded in achieving this goal in the mid-1990s, using a variety of MDR cell lines and transfected cells as the source of protein (for a review, see Sharom 1997a). In general, expression in heterologous cells, such as bacteria, yeast and insect cells has proved to be problematical, and mammalian cells are most reliable as a source of active Pgp. However, expression in *Pichia pastoris* has been very successful, leading to the purification of milligram amounts of both wild-type and mutant Pgp (Lerner-Marmarosh et al. 1999).

Proteoliposomes containing reconstituted Pgp have also proved to be a powerful tool for characterization of the drug transport process (Sharom et al. 1993; Sharom et al. 1996; Shapiro and Ling 1995; Eytan et al. 1996; Ambudkar et al. 1998). They showed that transport is osmotically sensitive, active (generating a substrate concentration gradient across the bilayer of 5- to 6-fold), saturable at increasing substrate concentrations, and requires ATP hydrolysis. Other Pgp substrates and modulators block transport in a saturable manner. Real-time fluorescence-based assays can continuously monitor the transport of fluorescent substrates, such as Hoechst 33342 (H33342) (Shapiro et al. 1997) and tetramethylrosamine (TMR) (Lu et al. 2001b), allowing direct estimation of initial rates of transport. The true kinetic parameters for drug transport have also been obtained for both the transport substrate and ATP (Lu et al. 2001c).

ATPase activity of Pgp

ATP hydrolysis by Pgp takes place at the two NB domains located on the cytoplasmic face of the protein. The NB domains of all ABC proteins are characterized by Walker A and Walker B motifs, found in many proteins that bind ATP or GTP, and a signature C motif unique to the ABC superfamily. Mutational analysis has been very useful in defining the roles in catalysis of various amino acid residues in these three motifs (Loo and Clarke 1998; Frelet and Klein 2006). Studies with purified Pgp revealed that the protein displays high levels of constitutive ATPase activity, in the apparent absence of drug substrates. This behaviour is unusual; in most ATP-driven transporters, hydrolysis of ATP is tightly coupled to concurrent movement of substrate across the membrane. The basal ATPase activity of purified Pgp is as high as 3-5 $\mu\text{mol}/\text{min}/\text{mg}$ of protein, depending on the presence of lipids and detergents. The stoichiometry of ATP hydrolysis relative to drug transport is still a matter of controversy, mainly because of the high basal ATPase activity (Eytan et al. 1996; Ambudkar et al. 1997; Shapiro and Ling 1998a). Membrane-bound or purified Pgp shows a relatively high K_M for ATP hydrolysis, in the 0.4 mM range, and a divalent cation is also necessary (physiologically, this is Mg^{2+} , but Mn^{2+} and Co^{2+} also support ATP hydrolysis)(for a review, see Senior et al. 1995a). Sulfhydryl-modifying reagents, including N-ethylmaleimide, HgCl_2 , p-chloromercuribenzoate and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), inhibit Pgp ATPase activity by covalently modifying a Cys residue in the Walker A motif of each active site (Doige et al. 1992; al-Shawi et al. 1994; Loo and Clarke 1995a).

Ortho-vanadate (V_i), a phosphate analogue that inhibits several other ATPases, also inactivates Pgp in a reversible fashion. V_i can replace P_i in a single active site after ATP hydrolysis, leading to formation of a highly stable trapped complex that is thought to have the structural geometry of the transition state that exists transiently during ATP hydrolysis. The V_i -trapped complex, $\text{Pgp}\cdot\text{ADP}\cdot V_i\cdot M^{2+}$, has no ATPase activity, despite the fact that one active site is unoccupied (Urbatsch et al. 1995). Slow dissociation of V_i

from the active site, followed by dissociation of ADP, leads to full restoration of ATPase activity.

Drugs and modulators affect Pgp ATPase activity in a complex fashion (Borgnia et al. 1996). Many drugs show biphasic modulation of activity, with stimulation at low concentrations and inhibition at high concentrations. Some substrates only stimulate activity, while others only inhibit it. At present, there is no satisfactory explanation for the different patterns that are observed. The biphasic pattern might arise from the presence of two drug-binding sites, a high affinity stimulatory site and a lower affinity inhibitory site (Gottesman et al. 1996). Results have been variable between different research labs, and the presence of various lipids and detergents also appears to affect the drug interaction patterns (Urbatsch and Senior 1995; Ambudkar 1995).

Pgp as a hydrophobic vacuum cleaner and drug flippase

Classical membrane pumps, such as lactose permease or the Na^+, K^+ -ATPase, transport polar or charged substrates across the membrane, by moving the substrate through a path within the protein that is lined with polar residues (Fig. 2). In this way, the substrate does not contact the hydrophobic interior of the lipid bilayer, which is a thermodynamically unfavourable event. However, Pgp substrates are relatively non-polar, and they are known to partition into lipid bilayers and accumulate to high concentrations. It was suggested that Pgp may function as a "hydrophobic vacuum cleaner" (Fig. 2), binding non-polar compounds that partition into the membrane and expelling them into the extracellular medium (Higgins and Gottesman 1992). Pgp can also be envisaged as a drug "flippase", moving its substrates from the cytoplasmic membrane leaflet to the extracellular leaflet, where they can partition into the aqueous phase (Fig. 2). This idea is supported by substantial experimental evidence (Sharom 1997a), including localization of the drug-binding sites of Pgp to the cytoplasmic membrane leaflet (Shapiro and Ling 1997a; Shapiro and Ling 1998b; Ferry et al. 2000; Qu and Sharom 2002; Lugo and Sharom 2005b).

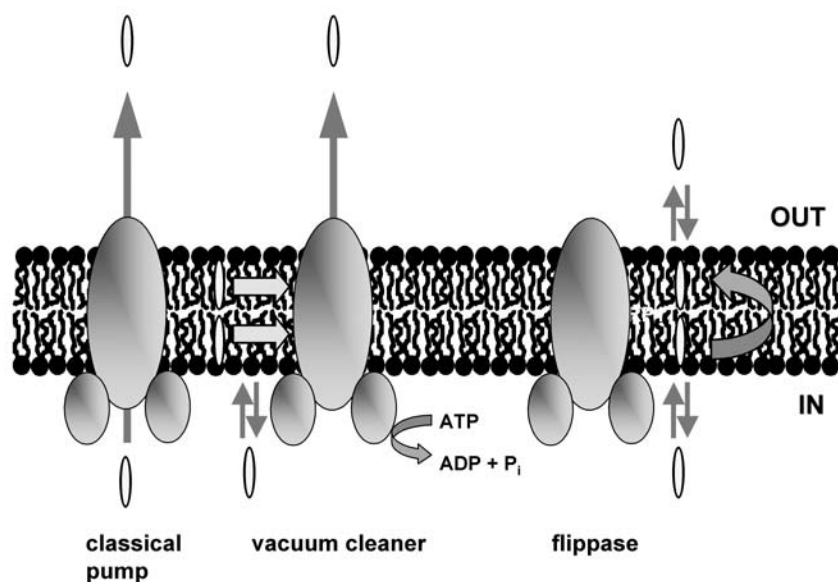


Figure 2 Classical pump, vacuum cleaner and flippase models of Pgp action. Classical pumps transport a polar substrate from the aqueous phase on one side of the membrane to the aqueous phase on the other side through a hydrophilic path formed by the TM regions of the protein. In the vacuum cleaner model, drugs first partition into the lipid bilayer, and interact with Pgp within the membrane. They are subsequently effluxed into the aqueous phase on the extracellular side. In the flippase model, drugs partition into the membrane, interact with the drug binding pocket in Pgp within the cytoplasmic leaflet, and are then translocated to the outer membrane leaflet, where they can partition into the extracellular aqueous phase.

Fluorescence approaches for studying Pgp structure and function

Fluorescence spectroscopic techniques have found increasing application to membrane proteins in recent years. They have the advantage of high sensitivity, so that only small amounts of protein are required, and can be used to explore many different aspects of protein structure and function. It is very difficult to study drug interactions with Pgp by classical biochemical techniques because of their nonpolar nature. Fluorescence approaches have circumvented these problems, and revealed important information that is not easy to obtain by other means. Using purified Pgp, it is possible to covalently link extrinsic fluorophores to specific residues on the protein, where they act as reporter groups. One very useful target in this approach has been Cys residues. The first fluorescence study of Pgp linked 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS) to the Cys residue that is present in the Walker A motif of each ATPase

active site (Liu and Sharom 1996). More recent studies have used intrinsic Trp fluorescence to examine the behaviour of the unmodified protein (Liu et al. 2000; Sonveaux et al. 1999). Pgp substrates include many fluorescent dyes, such as rhodamines, H33342 and LDS-751, and they have also been used in transport studies, and to probe the drug-binding pocket. Finally, Pgp can bind a variety of fluorescent nucleotides, including trinitrophenyl(TNP)-ATP, MANT-ATP and ϵ -ATP, which can yield useful information about the nucleotide binding site. Fluorescence studies have provided information on the following aspects of Pgp structure and function (for a review, see Sharom et al. 2001):

1. Binding of substrates and nucleotides; identification of substrates, quantitation of substrate and nucleotide binding affinities, estimation of nucleotide binding stoichiometry.
2. Probing of the local environment of the drug-binding pocket and the nucleotide binding site.
3. Transport kinetic studies; initial rates of drug

transport in real time, kinetic parameters of transport, flip-flop rates of fluorescent phospholipid and glycosphingolipid derivatives.

4. Characterization of conformational changes taking place during the catalytic and transport cycle.
5. FRET (fluorescence resonance energy transfer) studies; estimation of the distances between domains and regions of the protein, generation of a low resolution map of Pgp architecture.

Structure of Pgp

The topology of Pgp in the membrane was established using molecular biological approaches such as insertion of glycosylation sites (Kast et al. 1996) and Cys mutations (Loo and Clarke 1995b). It consists of two homologous halves, each with 6 TM segments and a cytosolic NB domain (Fig. 3A). Mutagenesis studies revealed that the drug-binding site is formed by the TM regions of both halves of Pgp, especially TM4, 5 and 6 in the N-terminal half, and TM9, 10, 11 and 12 in the C-terminal half (Loo and Clarke 2005). Substrates gain entry to this site from within the membrane. The NB domains of ABC proteins are highly conserved, and share several common motifs, including the Walker A and B motifs that are found in other ATPases, and the ABC signature, or C motif, that is unique to the protein family.

High resolution crystal structures for two bacterial ABC proteins, the DNA repair enzyme Rad50cd (Hopfner et al. 2000) and the vitamin B12 importer BtuCD (Locher et al. 2002), showed that the two NB domains were closely associated, forming a dimeric structure in which the Walker A and B motifs of one NB domain and the C motif of the partner NB domain formed the ATP binding sites. This type of arrangement had previously been predicted by Jones and George (1999) based on biochemical and sequence considerations. This structure, now known as a nucleotide sandwich dimer, was also observed in the catalytically inactive E171Q mutant of the isolated NB domain of the ABC protein MJ0796, where two molecules of ATP are bound at the dimer interface (Smith et al. 2002). The process of tight dimerization of the

NB domains, induced by ATP binding, may be an important step in the catalytic cycle of the ABC proteins. Three high resolution structures have been published for MsbA, a homodimeric bacterial lipid A flippase (Chang and Roth 2001; Chang 2003; Reyes and Chang 2005). Far from clarifying the structure of this protein, three quite different dimer arrangements were observed, adding more uncertainty to the ABC protein field (Davidson and Chen 2005). The transmembrane regions of the MsbA dimer showed 12 TM helices, and it has thus been a popular basis for building homology models for Pgp (Stenham et al. 2003). However, the three MsbA structures were withdrawn in late 2006 when a data-processing error was discovered. A recent high resolution structure of the ABC protein Sav1866, shows a quite different arrangement of the 12 TM helices, in which the two halves are entwined with each other (Dawson and Locher 2006), raising doubt as to which structural model of the TM domains might best apply to eukaryotic proteins such as Pgp.

High resolution structural information for Pgp is still lacking. A low resolution structure determined by electron microscopy (EM) using single particle analysis showed a large 5 nm central pore in the protein, which was closed at the cytoplasmic side, and two widely separated lobes that were thought to be the NB domains (Rosenberg et al. 1997). This structure disagreed with biochemical studies and the other ABC protein structures described above, which showed close association between the NB domains. Further studies indicated that nucleotide binding causes a repacking of the TM regions of Pgp, opening the central pore to allow access of hydrophobic drugs directly from the lipid bilayer (Rosenberg et al. 2001; 2003), leading to the proposal that ATP binding, rather than hydrolysis, drives the conformational changes associated with transport. The vanadate-trapped complex of Pgp displayed a different conformation, suggesting that rotation of TM α -helices takes place during the catalytic cycle.

In contrast to these structures, a low resolution EM study of 2D crystals of Pgp, showed that the molecule was compact, with closely associated NB

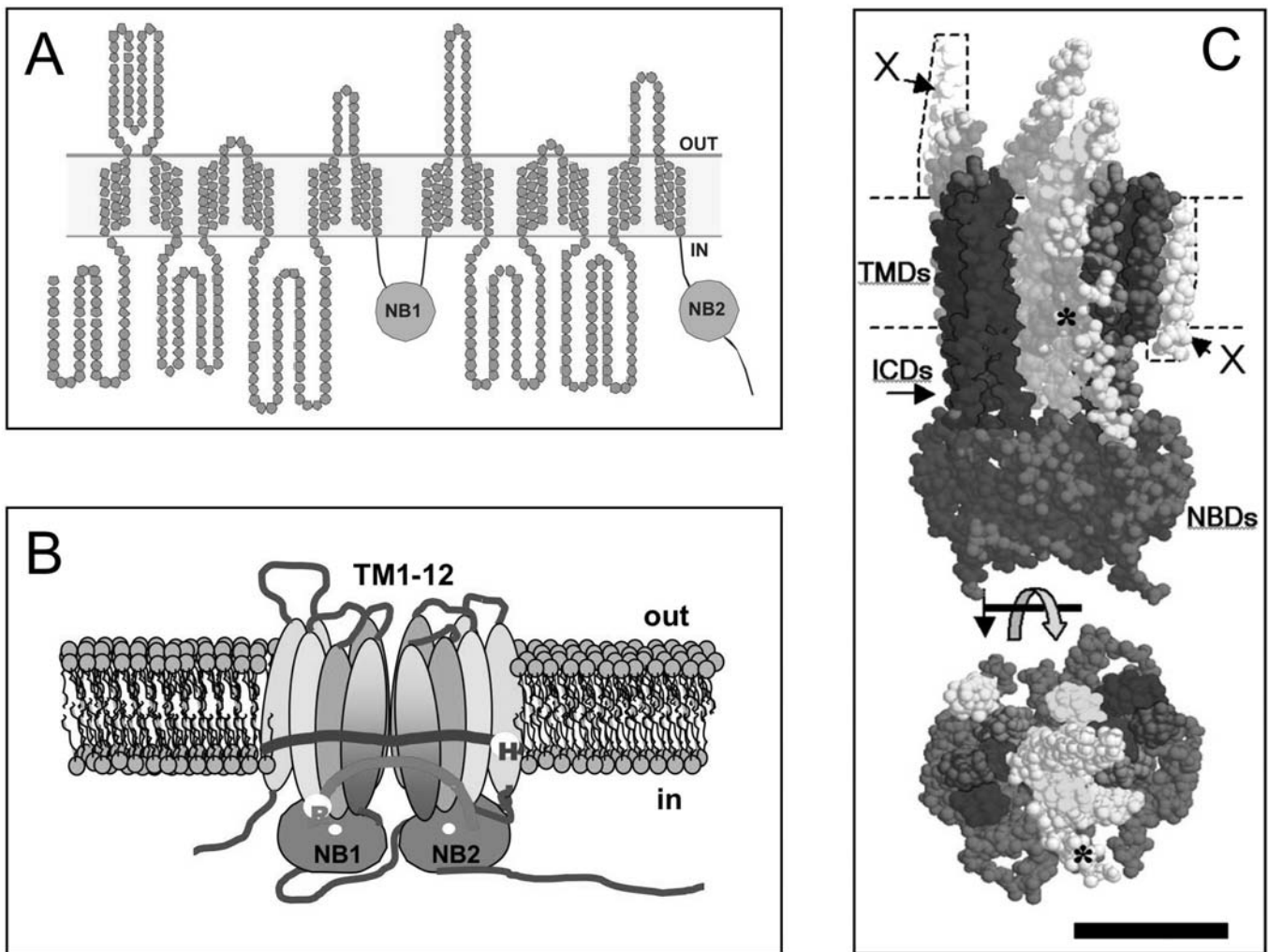


Figure 3 Topology and structure of Pgp. (A) Pgp is proposed to consist of two equivalent halves, each with 6 TM segments and a cytoplasmic NB domain. (B) Low resolution structural model of Pgp generated using FRET measurements of the distances separating key regions of the protein (Lugo and Sharom 2005b). The curves labelled H and R represent the boundaries of the binding sites for the dyes H33342 and LDS-751, respectively, as estimated by FRET analysis. (C) Medium resolution structural model of Pgp obtained from cryo-EM studies (Rosenberg et al. 2005). Top: a side view of the protein is shown with the NB domains at the bottom. The 12 putative TM α -helices are arranged in a pseudo-symmetrical relationship. * indicates the location of one of four helices without an obvious symmetry relationship; another (indicated by X) has a poorly defined location, and the other two are on the extracellular face of Pgp. Bottom: a view of Pgp looking down on the TM helices from the extracellular side of the membrane. The dashed lines indicate the putative boundary of the lipid bilayer (scale bar = 5 nm). Adapted from Rosenberg et al. (2005) with permission.

domains (Lee et al. 2002). FRET studies in which two different fluorescent probes were attached to the Walker A Cys residues also suggested that the two active sites are closely associated, compatible with the sandwich dimer model (Qu and Sharom 2001). FRET mapping of various intramolecular distances within the Pgp molecule led to a low resolution model of the protein structure (Lugo and Sharom 2005b)(Fig. 3B). A medium resolution EM structure of Pgp appeared recently (Fig. 3C), and showed closely associated NB domains and the existence of 12 TM seg-

ments, supporting the proposed topology (Rosenberg et al. 2005).

Fluorescence approaches for studying binding of nucleotides and drugs to Pgp

Photoaffinity labelling has been a popular approach to demonstrate interaction of azido-analogues of nucleotides and drugs with Pgp, both in native membrane vesicles and

purified protein. Competition experiments with unlabelled drugs and modulators have given a crude indication of relative binding affinity. However, technical limitations, such as low labelling stoichiometry and variable labelling efficiency, complicate interpretation of the results, and quantitation of binding affinity is not possible using this approach.

Classical biochemical approaches using equilibrium binding are unable to quantitate ATP binding to Pgp, since it is of relatively low affinity. Fluorescence approaches can measure equilibrium binding without the need to separate Pgp-bound nucleotide from free nucleotide. Nucleotide binding was first characterized using MANS-labelled Pgp. ATP binding resulted in saturable quenching of the MANS fluorescence, likely as a direct result of a change in the local environment of the probe, which is located close to the site of ATP binding (Liu and Sharom 1996). Fitting of the data to an equation for binding to a single site led to an estimate for K_d of ~ 0.4 mM, similar to the K_M for ATP hydrolysis.

More recently, an intrinsic Trp quenching approach has been used to quantitate nucleotide binding. Trp residues are highly sensitive to their local environment, so that binding of substrates often leads to quenching of their fluorescence emission. Pgp has 11 Trp residues, but the blue-shifted nature of the fluorescence spectrum indicates that only a few, likely those in the TM segments, contribute to Pgp emission. Trp fluorescence was shown to be saturably quenched by binding of both unmodified and TNP-labelled nucleotides (Liu et al. 2000). Quenching of Trp residues appeared to arise from FRET to the bound nucleotide, suggesting that the NB domains are packed relatively closely with the TM regions of the protein.

Interaction of fluorescent nucleotides with Pgp results in a large enhancement of the fluorescence emission relative to aqueous solution, because of the relatively non-polar nature of the binding site (Liu and Sharom 1997). Work by the group of Di Pietro showed that in addition to this feature, intrinsic Trp fluorescence, could be used to monitor binding of fluorescent nucleotide derivatives to

the expressed C-terminal NB domain, which contains a single Trp residue (Baubichon-Cortay et al. 1994). The expressed N-terminal NB domain was also able to bind fluorescent nucleotides (Dayan et al. 1996). Binding of TNP-ATP/ADP to Pgp is of higher affinity compared to unmodified nucleotides ($K_d \sim 35-45$ μ M), because of additional interactions between the non-polar TNP group and the binding pocket. Fluorescence measurements were also used to determine the stoichiometry of TNP-nucleotide binding. Native Pgp binds two molecules of nucleotide under the conditions that exist in the cytosol (Qu et al. 2003b). V_i -trapped Pgp, on the other hand, binds only one molecule of nucleotide at the unoccupied active site. A recent report of binding of a spin-labelled nucleotide derivative to Pgp supported these conclusions, showing a binding stoichiometry of 2 and a binding affinity of 0.2 mM (Delannoy et al. 2005).

Measuring binding of drugs and modulators to Pgp is especially challenging because of their lipophilic nature and high propensity to partition into membranes. Equilibrium binding studies have been carried out to quantitate the drug binding affinity and capacity of Pgp, but they are technically challenging, and only a few drugs can be studied using this approach (Taylor et al. 1999; Martin et al. 2000). Fluorescence approaches have been invaluable in demonstrating a direct interaction between Pgp and many different drugs, and also in quantitating their binding affinity. Studies using MANS-Pgp revealed that binding of drugs and modulators to the substrate binding pocket resulted in quenching of MANS in the ATPase active site. These results suggested the existence of conformational communication between the drug-binding site(s) in the TM regions and the active site in the NB domain, which alters the local environment of the MANS probe. This conformational change is also reflected in the observed stimulation of ATPase activity by drugs and modulators. Quenching by drugs is saturable, and can be fitted to a binding equation to extract estimates of the binding affinity (Liu and Sharom 1996). The K_d values measured by fluorescence quenching for a large number of drugs cover 4 orders of magnitude, from 158 μ M for the low affinity substrate colchicine, to 37 nM

for paclitaxel, a high affinity substrate (Sharom et al. 1999; Sharom et al. 2001). The binding affinity of a drug or modulator (as measured by the K_d value from fluorescence experiments) is highly correlated with its ability to inhibit Pgp-mediated transport of ^3H -colchicine in native plasma membrane vesicles, suggesting that all substrates make use of a common pathway and transport mechanism (Sharom et al. 1998; Sharom et al. 1999).

Intrinsic Pgp fluorescence can be used to examine drug binding more directly. Trp residues are also quenched saturably by binding of drugs and modulators, and these experiments give estimates of binding affinity very similar to those obtained using MANS-Pgp quenching (Liu et al. 2000). The high degree of Trp quenching observed for some drugs suggests that these aromatic residues may be directly involved in binding substrates, perhaps via π - π stacking interactions. Aromatic residues are over-represented in the TM regions of Pgp, and their involvement in substrate recognition and binding by Pgp was previously suggested (Pawagi et al. 1994).

Several compounds, including H33342 and LDS-751, show greatly enhanced fluorescence emission on binding to the drug binding pocket of Pgp, which has hydrophobic character (see below). This phenomenon can also be used to directly quantitate their interaction with purified Pgp (Qu et al. 2003a; Lugo and Sharom 2005a; Lugo and Sharom 2005b). The measured binding affinities are in agreement with those estimated by both MANS quenching and Trp quenching; thus the three different fluorescence approaches for measuring drug binding to Pgp all yield very similar quantitative information.

Probing the drug-binding pocket of Pgp

Much effort has been expended to try to understand how Pgp can interact with such a large number of structurally dissimilar compounds. Attempts to develop quantitative structure-activity relationships (QSAR) for Pgp substrates and modulators, to link their chemical and physical properties with their biological activity, have been fraught with difficulties. The best description of a substrate

appears to involve a set of structural elements required for interaction of a compound with Pgp (Seelig 1998; Seelig and Landwojtowicz 2000; Cianchetta et al. 2005), consisting of two or three electron donors (hydrogen bond acceptors), or hydrophobic units, arranged in a fixed spatial separation. Pajeva and Wiese have proposed a pharmacophore model consisting of two hydrophobic units, three hydrogen bond acceptors, and one hydrogen bond donor (2002). There have been suggestions of the existence of multiple separate drug-binding sites, but the current consensus is that Pgp probably possesses a single, large, flexible drug-binding pocket. Drugs are believed to interact with the amino acid residues lining this pocket by an "induced-fit" type of mechanism, involving multiple Van der Waal's and hydrophobic interactions which can be different for each compound. The principles of such multidrug binding have been well established for soluble bacterial transcriptional regulators (Schumacher and Brennan 2002), and it is likely that they also apply to mammalian multidrug transporters (Zheleznova et al. 2000; Loo et al. 2003). The drug-binding pocket is located within the membrane-bound regions of Pgp, and is made up from several TM segments. It appears to be funnel-shaped, and is narrower at the cytoplasmic side, where TM2/11 and TM5/8 come together (Loo and Clarke 2005). It may lie at the interface between the N- and C-terminal halves of the protein (Pleban et al. 2005).

There appear to be two "functional" transport sites within Pgp; the R-site, which interacts preferentially with rhodamine 123, and the H-site, which interacts preferentially with H33342 (Shapiro and Ling 1997b). These two sites interact with each other allosterically in a complex fashion. Binding of a drug to the H-site stimulates transport of an R-site drug, while inhibiting transport of other H-site drugs. Binding of an R-site drug has the reciprocal effect; it stimulates transport of an H-site drug, while inhibiting transport of other R-site drugs. It is not clear whether these functional sites have distinct physical locations within the protein.

What is the nature of the drug-binding pocket of Pgp? Fluorescence spectroscopy can again provide some answers that cannot be obtained by other

means. Suggestions that the drug-binding pocket was open to an aqueous chamber (Loo et al. 2004; Rosenberg et al. 2001) was not supported by fluorescence characterization. Large increases in fluorescence emission intensity, coupled with a blue shift in the emission wavelength, indicated that drugs bound to both the H-site and the R-site are in a very hydrophobic environment, with a polarity lower than that of chloroform (Qu and Sharom 2002; Lugo and Sharom 2005b). A detailed biochemical characterization of the R-site was recently carried out by our laboratory, using the fluorescent drug LDS-751 (Lugo and Sharom 2005a). We found that two drugs, rhodamine 123 and LDS-751, which compete with each other for transport by Pgp, can both bind to this site simultaneously. However, they compete with each other non-competitively, rather than competitively. Estimation of the binding parameters for each drug alone and in the presence of the other indicated that they have a 5-fold negative effect on each other's binding; bound LDS-751 reduces the binding affinity of rhodamine 123 by a factor of 5, and vice versa. The two drugs may bind to different overlapping regions, or mini-pockets, within the large flexible binding site. Steric interference would account for the observed reciprocal negative effect of each drug on the binding of the other.

Role of the lipid bilayer in Pgp function

The functioning of the Pgp molecule is inextricably linked to the lipid bilayer in which it is embedded, and from which it obtains its substrates (for a review, see Ferté 2000). The study of how Pgp is modulated by the membrane required reconstitution of the purified protein into bilayers of defined phospholipids with specific biophysical properties (Romsicki and Sharom 1997). Lipid is essential for catalytic function of the NB domains (Doige et al. 1993), and lipids also influence both basal ATPase activity and its stimulation or inhibition by drug substrates (Urbatsch and Senior 1995; Sharom 1997b). Purified Pgp was found to retain ~55 tightly bound phospholipids; whose removal led to complete loss of function (Sharom et al. 1995). The presence of cholesterol has also been

linked to Pgp function, both directly in model systems (Rothnie et al. 2001), and also indirectly in intact cells (Wang et al. 2000; Garrigues et al. 2002; Troost et al. 2004). Pgp is functional in a lipid environment that mimics sphingolipid/cholesterol-rich microdomains or "lipid rafts" (Modok et al. 2004), and various reports have suggested that the protein may be located in specialized regions of the plasma membrane in intact cells (Demeule et al. 2000; Hinrichs et al. 2004; Radeva et al. 2005; Orłowski et al. 2006).

The NB domains, which are usually thought of as separately-folded soluble domains, are surprisingly affected by the fluidity of the bilayer in which the protein is reconstituted. The kinetic parameters of ATP binding and ATP hydrolysis by Pgp differ, depending on the phase state of the host lipids (Romsicki and Sharom 1998). Lipids may modulate the function of the NB domains of Pgp indirectly by interacting with the TM regions of the protein, or the NB domains themselves may interact with the bilayer surface.

The bilayer also plays a major role in the interaction of drugs with the protein. Given their high lipid-water partition coefficients, P_{lip} , Pgp substrates will accumulate to very high levels in the membrane (Romsicki and Sharom 1999; Regev et al. 2005; Siarheyeva et al. 2006; Gatlik-Landwojtowicz et al. 2006). The actual drug concentration in the membrane may be 300- to 2000-fold higher than the concentration added to the aqueous phase. Thus Pgp may have a relatively low intrinsic affinity for its substrates; the role of the membrane is to concentrate the drug for presentation to the protein. The rate of transport of H33342 was shown to be proportional to its bilayer concentration, confirming that this substrate is removed from the membrane (Shapiro et al. 1997). The lipid composition of the host bilayer affects the ability of drugs to partition into the membrane. It was found that the apparent K_d value for drug binding correlated with the P_{lip} values (see Fig. 4); the higher the partitioning of a drug into the lipid, the lower the measured K_d value, in other words, the higher the apparent binding affinity (Romsicki and Sharom 1999). The interaction of a drug with

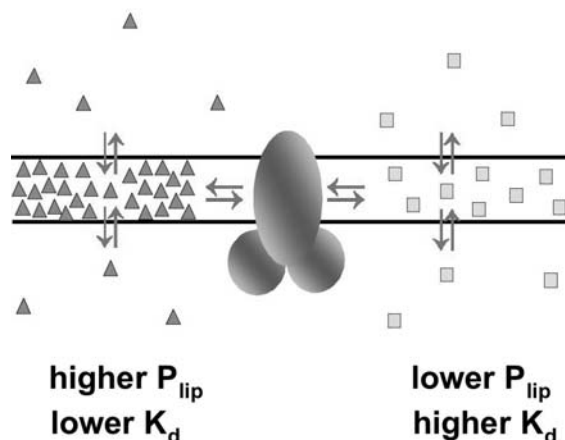


Figure 4 The binding affinity of Pgp for a particular drug substrate, K_d , is related to its lipid-water partition coefficient, P_{lip} . A drug with a high value of P_{lip} (left side) will accumulate to a high concentration within the membrane, favouring binding to Pgp and resulting in a low apparent K_d . In contrast, a drug with a low value of P_{lip} (right side) will have a lower membrane concentration, and a higher apparent K_d .

Pgp is thus strongly modulated by its lipid partitioning ability. Omote and Al-Shawi (2006) recently conducted a molecular dynamics simulation of the lipid bilayer partitioning and transbilayer movement of drugs by Pgp. They proposed that drugs are initially located in the interfacial region of the cytoplasmic membrane face, and are expelled to the exterior by a solvation exchange mechanism.

The fluidity of the host membrane also affects Pgp-mediated drug transport. When Pgp was reconstituted into proteoliposomes of differing fluidity, it was found that the initial rate of TMR transport measured by a real-time fluorescence assay showed an unusual biphasic temperature dependence (Lu et al. 2001a). The transport rate was high in the rigid gel phase, reached a maximum at the melting temperature of the bilayer, and then declined in the fluid liquid crystalline phase. Partitioning of organic compounds into lipid bilayers shows a similar pattern, suggesting that the rate of drug transport may be dominated by drug partitioning into the membrane.

Lipid flippase activity of Pgp

An early proposal suggested that Pgp might function as a drug “flippase”, moving hydrophobic mol-

ecules from the inner to the outer leaflet of the membrane (Higgins and Gottesman 1992). Support for this idea came later, from the finding that the highly homologous protein ABCB4 is a flippase for PC, exporting it from the apical membrane of the liver canalicular cells into the bile (Ruetz and Gros 1994; Smith et al. 1994). Further studies in intact cells provided evidence that Pgp can translocate both short chain phospholipids and glycosphingolipids from the inner to the outer leaflet of the plasma membrane (van Helvoort et al. 1996; van Meer et al. 1999). The use of proteoliposomes containing reconstituted Pgp provided direct evidence of this flippase activity, and allowed its characterization (Sharom et al. 2005). Romsicki and Sharom (2001) used a fluorescence quenching technique to show that Pgp could flip a variety of NBD-labelled phospholipids and sphingomyelin, and this work was extended by Eckford and Sharom (2005), who found that fluorescent analogues of the simple glycosphingolipids, galactosyl- and glucosylceramide were also flipped at high rates. The process of lipid flipping resembles drug transport in that it requires ATP hydrolysis and is inhibited by ortho-vanadate. Drugs and modulators are able to compete with membrane lipids for flipping, and their inhibitory potency is highly correlated with their Pgp binding affinity as measured by fluorescence approaches, suggesting that the two types of molecule follow a similar route through the protein.

The fluorescent phospholipids, glycolipids and sphingolipids commonly used in flippase studies in intact cells and model systems usually (but not always) have one short acyl chain. Whether normal membrane lipids with two long acyl chains are good substrates for Pgp is still not clear, since it is very difficult to test flipping of normal unlabelled lipids in model systems. However, Pgp was able to translocate a fluorescent PE derivative with two 16-carbon or 18-carbon acyl chains. In addition, a large number of ABC transporters appear to translocate natural membrane lipids and sterols, and this may be a side activity of all proteins in this family (Borst et al. 2000; Kálin et al. 2004; van Meer et al. 2006). It is possible that Pgp plays a physiological role in translocating glucosylce-

ramide from the cytoplasmic to the luminal side of the Golgi membrane, a process which must take place during glycolipid biosynthesis (Lala et al. 2000). Pgp thus appears to be a broad specificity, outwardly-directed flippase for a variety of lipid analogues. The ability of Pgp to transport lipophilic drugs may have evolved from its lipid flippase function, since its close relative, ABCB4, can apparently transport lipophilic drugs at a low level (Smith et al. 2000).

The catalytic cycle of Pgp

The mechanism of transport by ABC proteins involves two distinct, but coupled cycles. First there is the catalytic cycle whereby ATP is hydrolyzed; this comprises ATP binding, formation of a putative nucleotide sandwich dimer, hydrolysis of ATP, dissociation of P_i , and dissociation of ADP. The energy derived from this cycle is coupled to movement of substrate across the membrane, although there are opposing views as to whether the energy is provided by ATP binding, or ATP hydrolysis (Higgins and Linton 2004; Hanekop et

al. 2006). Drug transport by Pgp involves entry of the substrate into the binding pocket, conformational changes, and drug release. It is assumed that dissociation of drug on the membrane exterior involves re-orientation of a drug-binding site from the cytosolic side of the membrane (likely in the inner membrane leaflet) to the extracellular side (possibly the outer membrane leaflet), with a concomitant switch from high to low drug-binding affinity. For a comprehensive recent review on this topic, see Callaghan et al. (2006). The drug-binding site and the active sites in the NB domains must communicate with each other, likely via conformational changes, so that drug binding activates ATP hydrolysis and initiates the transport cycle.

The approach of vanadate trapping has led to some important insights into the ATPase catalytic cycle. For the myosin ATPase, such trapped complexes have provided very useful structural information (Smith and Rayment 1996). V_i is trapped in only one of the NB domains of Pgp after a single cat-

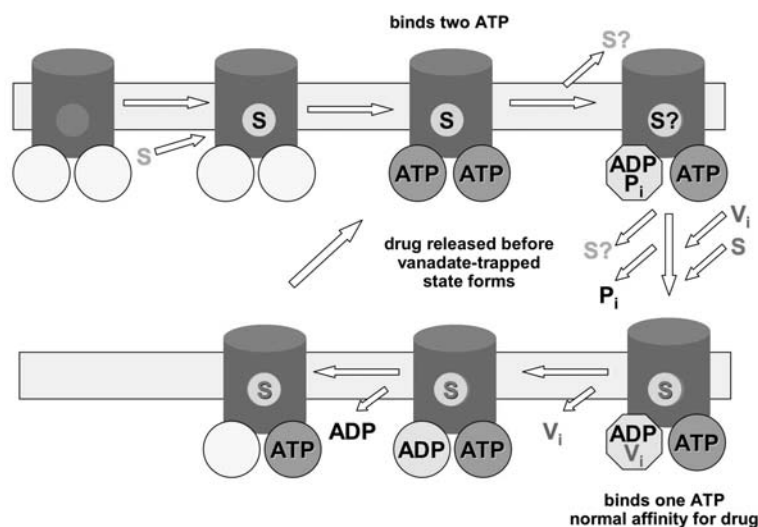


Figure 5 Vanadate trapping and proposed catalytic cycle of Pgp. The substrate, S, and two molecules of ATP bind to Pgp in a random order (Liu et al. 2000; Qu et al. 2003b). Following hydrolysis of ATP at one active site, dissociation of P_i and its replacement by V_i leads to formation of the stable vanadate-trapped complex, $Pgp \cdot ADP \cdot V_i \cdot M^{2+}$, which has no ATPase activity but retains one molecule of bound ATP (Qu et al. 2003b). Substrate is likely translocated across the membrane simultaneously with either ATP hydrolysis or dissociation of P_i , by switching of a high affinity cytoplasmic drug binding site to an outward-facing low affinity drug binding site. The vanadate-trapped complex has already regained high affinity drug binding (Qu et al. 2003a; Russell and Sharom 2006), and binds another substrate molecule. Following dissociation of ADP, rebinding of ATP to the vacant active site leads to another round of transport. According to the alternating sites hypothesis, ATP hydrolysis takes place at the other NB domain during this second round of transport (Senior et al. 1995b). It is not yet known how this cooperativity between the two active sites is achieved.

alytic turnover, yet all ATPase activity is lost (Urbatsch et al. 1995). Senior et al. suggested that the protein operates by an alternating sites mechanism, in which only one catalytic site can be in the transition state at any instant in time, and the two sites alternate in catalysis (Senior et al. 1995b). It is not yet known how this co-operation between the two active sites is achieved at the molecular level, or how ATP hydrolysis is coupled to drug transport (reviewed by Ambudkar et al. 2006). The alternating sites model implies that asymmetry between the two NB domains of Pgp must exist at some point during the catalytic cycle. Evidence for such asymmetry has been reported by Tomblin and Senior (Tomblin et al. 2005), who found a single occluded ATP molecule tightly bound in one NB domain of the double "catalytic carboxylate" mutant (E552A/E1197A). This catalytically-defective mutant conformation appears to represent a transient asymmetric intermediate. They proposed that after loose binding of two ATP molecules the sandwich dimer forms, and the tightly-bound nucleotide is then committed to hydrolysis and rapidly enters the transition state (Tomblin and Senior 2005).

The energy from ATP hydrolysis has been proposed to drive drug transport via relaxation of a high energy intermediate, with one ATP hydrolyzed for each drug molecule translocated (Senior et al. 1995b). There is still controversy as to whether one or two rounds of ATP hydrolysis are required in each transport cycle. An alternate model has been put forward by Sauna and Ambudkar (2001), in which two molecules of ATP are hydrolyzed per cycle, the first to transport the substrate molecule, the second to "re-set" the protein for another round of transport. The ATP binding stoichiometry, nucleotide binding affinity, and drug binding affinity are known at various stages of the catalytic cycle (Qu et al. 2003a; Qu et al. 2003b; Delannoy et al. 2005), and incorporation of these parameters into a proposed transport scheme is shown in Fig. 5. We have recently initiated rapid kinetic studies of the Pgp transport cycle, using fluorescence tools (Lugo et al. 2006), and we have also begun to measure thermodynamic constants for some of the steps (Lugo and

Sharom 2005a). More studies of this type will be needed to fully elucidate the transport mechanism of Pgp.

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