FEBS Letters 580 (2006) 1056–1063

Minireview

The translocation mechanism of P-glycoprotein

Richard Callaghan[a,*], Robert C. Ford[b], Ian D. Kerr[c]

[a] Nuffield Department of Clinical Laboratory Sciences, Level 4, John Radcliffe Hospital, University of Oxford, Oxford, OX3 9DU, United Kingdom
[b] Faculty of Life Sciences, Jackson's Mill, University of Manchester, P.O. Box 88, Sackville Street, Manchester, M60 1QD, United Kingdom
[c] Centre for Biochemistry and Cell Biology, School of Biomedical Sciences, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH, United Kingdom

Received 17 October 2005; revised 16 November 2005; accepted 16 November 2005

Available online 19 December 2005

Edited by Gerrit van Meer

Abstract **Multidrug transporters are involved in mediating the failure of chemotherapy in treating several serious diseases. The archetypal multidrug transporter P-glycoprotein (P-gp) confers resistance to a large number of chemically and functionally unrelated anti-cancer drugs by mediating efflux from cancer cells. The ability to efflux such a large number of drugs remains a biological enigma and the lack of mechanistic understanding of the translocation pathway used by P-gp prevents rational design of compounds to inhibit its function. The translocation pathway is critically dependent on ATP hydrolysis and drug interaction with P-gp is possible at one of a multitude of allosterically linked binding sites. However, aspects such as coupling stoichiometry, molecular properties of binding sites and the nature of conformational changes remain unresolved or the centre of considerable controversy. The present review attempts to utilise the available data to generate a detailed sequence of events in the translocation pathway for this dexterous protein.**

Keywords: P-glycoprotein; Membrane transport; Drug-protein interaction; Drug-binding

1. What steps are required in an active translocation pathway?

The ability to translocate multiple substrates/drugs is shared by many transporters, however, the underlying mechanisms are yet to be elucidated. Multidrug transporters have been implicated in conferring resistance to chemotherapy in cancer, malaria and bacterial infection [1–3]. P-glycoprotein (P-gp, ABC^{B1}) is an archetypal multidrug transporter of the ATP binding cassette (ABC) family and the translocation of drugs across the plasma membrane by P-gp is a complex function involving allosteric communication between a multiplicity of drug binding sites and the two nucleotide binding domains (NBDs) [4]. To fully understand the molecular mechanism of translocation of drugs by P-gp requires identification of the individual steps and characterisation of the intermediate conformational states of the protein in the pathway. The first stages of the process will involve the association of drugs to high affinity binding sites and of hydrolysable nucleotide to the NBDs. These two processes are inextricably coupled, as evidenced by the ability of drugs to stimulate ATP hydrolysis and the dependence of translocation on the presence of two functional NBDs [4–7]. For P-glycoprotein, biochemical information exists that is consistent with the structural information derived from other ABC proteins that during the translocation cycle the two NBDs are brought into close proximity – termed the NBD sandwich, or closed dimer [8]. In order to generate an efficient and rapid translocation of drug it requires that the binding sites also switch from high affinity (to promote drug association) to low affinity (to promote drug dissociation), alternately exposed to opposite sides of the bilayer. Once the drug has been released to the extracellular environment, the protein must be reset to the initial conformation. The resetting phase of the translocation cycle will involve further alteration to NBD:NBD interactions and reorientation of the TMDs to restore high affinity drug binding. Clearly the overall pathway for drug translocation can be sub-divided into a series of intermediate steps. Each one of these steps will represent transition of the protein between distinct conformational states. The individual conformations, or transition intermediates, are formed by events such as: (i) association of nucleotide, (ii) dimerisation of NBDs or (iii) release of Pi to name a few. The propensity for transition between the conformations is governed by thermodynamic considerations and each may be represented by an equilibrium constant and ΔG. Each of the major steps in the translocation cycle comprises multiple thermodynamic equilibria, which may occur in distinct sequence. A proposed model for the entire pathway leading to translocation of drug by P-gp is shown in Fig. 1. This model, which contains multiple chemical equilibria, will be referred to as the thermodynamic transition model and the aim of this review is to discuss the likely, or preferred, sequence of steps in the overall translocation pathway. This thermodynamic transition model is discussed with regard to the four key stages of translocation: (A) loading of P-gp, (B) high to low affinity switch in drug binding sites, (C) energy utilisation and (D) resetting to the basal conformation. These individual stages (A–D) of the overall pathway are represented by cubes within Fig. 1.

2. Drug binding is required to couple the NBDs and TMDs

Fig. 2 represents a subsection of Fig. 1 and describes the transition from basal (Hn) to drug and ATP bound ($^{d}HN^{ATP}$) protein. This transition consists of a series of chemical equilibria and may in principle occur via multiple routes. However,

*Corresponding author. Fax: +44 1865 221 834.
E-mail address: richard.callaghan@ndcls.ox.ac.uk (R. Callaghan).

0014-5793/$32.00
doi:10.1016/j.febslet.2005.11.083

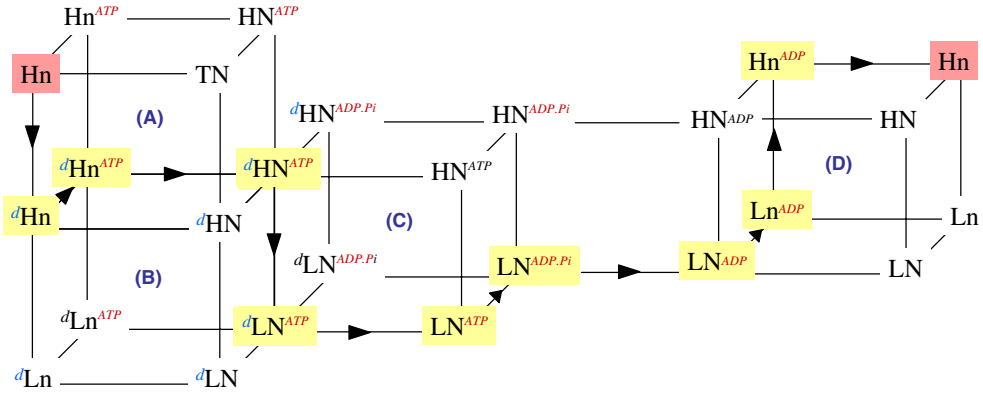

Fig. 1. A pharmacological model for drug translocation by P-gp. The entire translocation pathway has been depicted as a series of steps involving conformational transitions and interactions of the protein with drug and nucleotides. Each step represents an equilibrium reaction and the model displays all possible transitions (denoted by the straight lines). The drug binding sites within the TMD exist in the inward facing high affinity (H) or the outward facing low affinity (L) configurations. The NBDs are also in equilibrium between the separate (n) and engaged (N) conformations. Drug is denoted by "d" and the favoured series of transitions is depicted with the arrows and shaded transition intermediates. The starting point for the scheme is the drug and nucleotide free protein, with disengaged NBDs and a TMD with drug binding sites in the high affinity inward facing configuration (i.e., Hn). The lines containing an arrow correspond to the likely sequence of events in the translocation pathway as discussed in the main text. The cube denoted (A) refers to the loading of P-gp with drug and nucleotide; (B) depicts the high to low affinity reorientation of the drug binding sites; (C) depicts the energy utilisation step and (D) corresponds to the resetting phase.

only some of the steps are likely to occur and the precise route of the overall path will be dictated primarily by changes in free energy (ΔG) associated with individual equilibria. In this model, the basal conformation of P-gp comprises a high affinity inward facing drug binding site ('H') and the NBDs in a "disengaged" (open, 'n') configuration. During the transition to the fully loaded state, the NBDs assume close apposition to the classical sandwich (closed) dimer conformation. There is considerable experimental evidence to indicate that the NBDs are brought from an open to closed dimer association following the binding of nucleotide in a variety of ABC transporters. Crystal structure data for isolated NBDs indicates that both NBDs contribute to the binding interaction with ATP and that the nucleotide "bridges" the interface of the protein dimer [9–11]. Furthermore, structures generated for the bacterial lipid A transporter, MsbA, which is a P-gp homologue, propose extraordinarily large movements of the NBDs, including NBD dimerisation, during progression through the

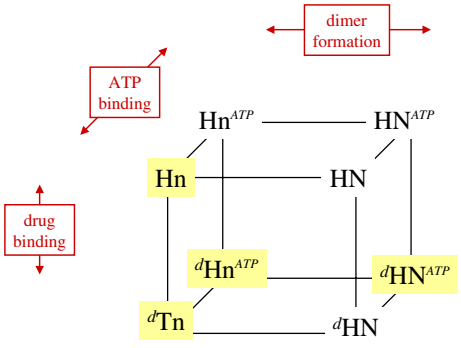

Fig. 2. Loading of P-gp with drug and nucleotide. A subsection of the thermodynamic transition model that corresponds to the initial stage of drug translocation; namely, loading of P-gp with drug and nucleotide. "H" corresponds to the drug binding sites within the TMD in the inward facing high affinity configuration. The NBDs are also in equilibrium between the separate (n) and engaged (N) conformations whilst drug is denoted by "d".

catalytic cycle [12–14]. For P-gp, the evidence of NBD interdigitation is more indirect; spectroscopic investigations demonstrate that the basal conformation has an initially low ATP binding affinity ($K_d \sim 0.5$ mM) [15] and numerous mutations within key motifs in the NBDs fail to impact significantly on nucleotide binding despite abrogating hydrolysis [16–19]. Such observations are consistent with a loose association of the NBDs prior to ATP binding. This initial loose binding is thought to eventually assume a higher affinity, presumably once the NBDs associate. Indeed, a murine P-gp isoform containing the double mutation of the Walker-B proposed catalytic glutamate residues (E522A/E1197A) displayed binding constants of $K_d = 9.2$ μM and 38 μM to the N- and C-terminal NBDs, respectively [20]. This increase in affinity from the initial $K_d = 500$ μM [15] provides support for a closed NBD dimer. The inability to readily disrupt this conformation is also consistent with a tight NBD association in P-gp. The association of ATP and movement of NBDs from open (n) to closed (N) dimer formation are depicted by the $Hn \rightarrow Hn^{ATP} \rightarrow HN^{ATP}$ transition sequence. This transition can occur in the absence of drugs as illustrated by the hitherto unexplained ability of P-gp to generate basal ATPase activity; i.e., ATP hydrolysis in the absence of transported substrate [21–24]. In fact, the transition sequence $Hn \rightarrow Hn^{ATP} \rightarrow HN^{ATP}$ is the likely sequence of events that supports basal ATPase activity; although this does not preclude hydrolysis from the disengaged conformation (i.e., $Hn^{ATP} \rightarrow Hn^{ATP}$) under basal conditions. The latter is unlikely to be the chosen route, particularly given the reported similarity of K_m(ATP) obtained for basal and drug stimulated ATPase activities [5,6,23,25].

The stoichiometric amount of ATP hydrolysed per translocation event remains undefined and the reported range varies considerably [22,26–29]. One of the major factors precluding elucidation of the ratio is the inherent difficulty in obtaining accurate transport rates for the hydrophobic substrates of P-gp in reconstituted proteoliposomes. It has been demonstrated that both NBDs are capable of binding nucleotide [5,7,17,24], and recent biochemical evidence indicates that although the open conformation of P-gp binds two nucleotides, the stable

NBD dimer binds ATP tightly with a stoichiometry of 1 mol/mol [30]. In addition, several "catalytically deficient" P-gp mutant isoforms have been demonstrated to trap nucleotide at a ratio of 1 mol/mol protein [31]. Numerous structural and molecular modelling investigations with bacterial members of the ABC transporter family in addition to P-gp suggest that only a single ATP molecule is trapped during a translocation cycle [32–34]. Moreover, the long established "alternating site" model of ATP hydrolysis by P-gp indicates that only a single ATP molecule is hydrolysed per transport event. An alternative model in which two ATP molecules are hydrolysed in order to transport [^{125}I]iodoarylazidoprazosin [35] has been presented. Unfortunately, the concentrations of drug and nucleotide used were only a small fraction of their respective K_d concentrations, thereby making quantitative predictions of stoichiometry less rigorous. The thermodynamic transition model may readily be expanded to incorporate two bound ATP molecules. In the present review, for simplicity and weight of available evidence, a single bound molecule of ATP is depicted and corresponds to the nucleotide that is hydrolysed during drug translocation.

Is the sequence affected by the presence of drug substrate? In order for drugs to affect ATP hydrolysis a communication pathway from binding sites in the TMDs and the NBDs is implicit. Biochemical data demonstrating that ATP binding to NBDs produces conformational changes in the TMDs has been obtained from numerous studies (outlined in Table 1) including (i) accessibility of cysteine residues introduced into TM6 [36], (ii) altered quenching of intrinsic tryptophan residues [37], (iii) differential protease digestion of P-gp [38,39], (iv) modified fluorescence of probes incorporated into the NBDs [15], (v) altered accessibility of an extracellular antibody epitope [40,41] and (vi) directly through visualisation of structural rearrangements [42–44]. A consistent observation from several of these studies is that the presence of transported substrates modified the magnitude and/or nature of the conformational change produced by nucleotide. Moreover, these allosteric communications between NBDs and TMDs produce functional perturbation that manifests as an acceleration of ATP hydrolysis [6].

The mechanism of this acceleration is not fully understood and drugs could alter one or more of the equilibria in the multistep ATPase activity of P-gp. For example, drugs could conceivably accelerate either engagement of NBDs ($Hn^{ATP} \rightarrow HN^{ATP}$), or dissociation of ADP post-hydrolysis. The latter occurs late in the translocation pathway, and drug induced acceleration at this stage seems intuitively unlikely. Indeed, studies by Martin et al [45,46], propose that drug is likely to have dissociated from the transporter prior to generation of the HN^{ADP} intermediate and thus is unlikely to affect the dissociation rate of ADP (Sections 3 and 4). By contrast, drug binding is likely to stimulate the overall rate of ATP hydrolysis by promoting engagement of the NBDs; i.e., the rate of the $^{d}Hn^{ATP} \rightarrow {}^{d}HN^{ATP}$ transition. Tombline et al. [31] have provided strong support using catalytically deficient P-gp isoforms in which several drugs accelerated the rate of nucleotide trapping within the closed NBD conformation. The kinetics of the $^{d}Hn^{ATP} \rightarrow {}^{d}HN^{ATP}$ transition will need to be significantly more rapid than the $Hn^{ATP} \rightarrow HN^{ATP}$ transition in order for P-gp to efficiently couple drug translocation with ATP hydrolysis. Slow kinetics of the $Hn^{ATP} \rightarrow HN^{ATP}$ conversion in the absence of drug substrate is responsible for the "sluggish" rate of ATP hydrolysis during basal ATPase activity.

Consequently, the reactions shown in the thermodynamic transition model above (initial binding events) are associated with "loading" of the transporter and comprise three key elements; drug association, ATP association and formation of the closed NBD dimer. The precise order of events is likely to follow the sequence $Hn \rightarrow {}^{d}Hn \rightarrow {}^{d}Hn^{ATP} \rightarrow {}^{d}HN^{ATP}$ as shown by the yellow boxes in the model. Moreover, the acceleration of NBD dimer formation by transported substrates is an important step in the coupling process.

3. ATP binding provides the initial impetus for translocation

Once P-gp has been "loaded-up" with substrate and nucleotide, the process of binding site reorientation and drug release begins. Initial hypotheses proposed that hydrolysis of ATP would provide the impetus for translocation. However, ATP binding, or binding of non-hydrolysable analogues such as adenyl-5′-yl imidodiphosphate (p[NH]ppA) and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), per se has been widely demonstrated to induce conformational changes in P-gp from the NBDs to TMDs and even extracellular loops. Table 1 provides a summary of the numerous investigations that have demonstrated these inter-domain communications. Molecular models of P-gp, based upon structural information [47] suggest that

Table 1
Nucleotide binding and/or hydrolysis events are associated with interdomain conformational changes in P-gp

Technique	Conformational change upon ATP binding/hydrolysis	Domain:domain communication identified	Reference
Electron microscopy[1,2]	Altered structure of the TMDs at medium resolution	NBD ↔ TMD	[42,43]
Fluorescence quenching[3]	Reduced fluorescence of MIANS-labelled P-gp (NBD-localized probe)	NBD ↔ drug binding sites	[51]
H/D exchange[3]	Faster kinetics of deuterium exchange	Global conformational change	[65]
Intrinsic fluorescence[1,2]	Increased quenching of tryptophan fluorescence	Global conformational change	[37]
Limited trypsin digest[1,2]	Enhanced resistance to trypsin digestion	NBD ↔ ECL	[38,66]
Pharmacology[1]	Reduced affinity for drugs	NBD ↔ drug binding sites	[45,46]
Photolabelling[4]	Reduced photo-labelling efficiency	NBD ↔ drug binding sites	[21,35,63]
Site-directed labelling and crosslinking[1,2]	Altered packing and orientation of TM helices	NBD ↔ individual TM helices	[36,48]
UIC2 binding[1]	Reduced exposure of the UIC2 epitope	NBD ↔ ECL	[33,67]

Notes. Where marked [1] a pre-hydrolytic state (e.g. bound non-hydrolysable nucleotide) was associated with a conformation distinct from the nucleotide free form. Where marked [2] addition of hydrolysable nucleotide, or trapping of a post-hydrolytic state, enabled detection of a distinct change in conformation compared to [1]. Where marked [3] the response to hydrolysable nucleotides alone has been reported. Where marked [4] nucleotide hydrolysis, as opposed to nucleotide binding, was required to identify a conformation distinct from the nucleotide free form.

these conformational changes are transmitted over distances greater than 50 Å. For example, cryo-electron microscopy of 2D-crystals of P-glycoprotein in nucleotide free and non-hydrolysable analogue bound states demonstrates altered architecture of the TMDs [43,44]. Additional studies have been able to identify specific TM segments (α-helices) that undergo changes in environment or spatial organisation in response to nucleotide binding. Some contrasting information has been obtained in studies by Loo and Clarke, which have mapped cross-linking patterns of P-glycoprotein isoforms containing two introduced cysteine residues in specific TM segments [48–50]. For the most part, the pattern of cross-links is identical in the nucleotide free and p[NH]ppA bound states [49,50], although there are some ATP-binding dependent conformational changes documented by this group [48]. Fluorescence studies of single cysteine containing isoforms of P-glycoprotein are also consistent with localised changes in the tilt of TM helix 6 subsequent to ATP binding [36].

However, few of these studies are able to provide a description of the molecular alterations in the drug binding sites induced by ATP binding. The possible functional changes (i.e., transition intermediate formation) to P-gp in response to ATP binding are summarised in the thermodynamic transition model shown in Fig. 3. Indeed, only pharmacological measurements of drug:P-gp interactions are able to directly relate changes in the affinity of drug interaction with the ATP bound state. In contrast, fluorescence quenching measurements [51], and photo-affinity labelling studies [21] provide only indirect evidence of the change in drug binding in the presence of nucleotide. Equilibrium radioligand binding assays demonstrate that non-hydrolysable nucleotides effected a reduction in the fraction of a transported substrate (vinblastine) bound to P-gp [45]. This reduction correlated with an almost 10-fold decrease in the affinity of vinblastine for P-gp in the p[NH]ppA bound conformation (from 80 nM to 730 nM [46]). Significantly, the vanadate-trapped conformation of P-gp showed identical affinity for vinblastine as the pre-hydrolytic (p[NH]ppA bound) state, demonstrating that the impetus for altering the drug binding site is derived from nucleotide binding, rather than nucleotide hydrolysis [46]. This may be interpreted as a reorientation of the vinblastine binding site to the

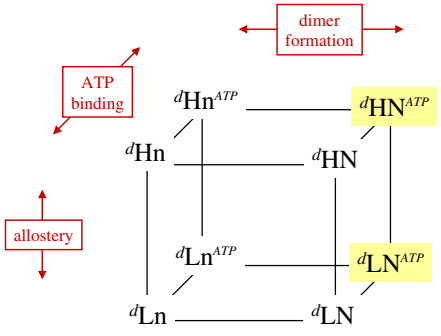

Fig. 3. Switch in affinity and orientation of the drug binding sites. A subsection of the thermodynamic transition model that depicts the switch in affinity and orientation of the drug binding sites within the TMDs. "H" corresponds to the drug binding sites within the TMD in the inward facing high affinity configurations. "L" corresponds to the drug binding sites within the TMD in the outward facing low affinity configuration. The NBDs are in equilibrium between the separate (n) and engaged (N) conformations whilst drug is denoted by "d".

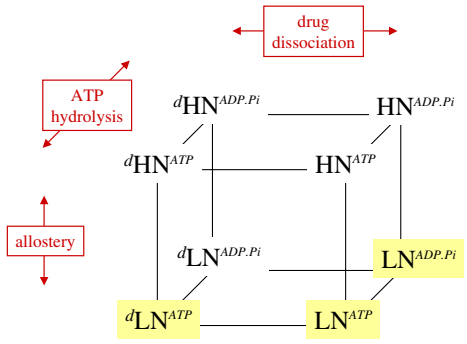

Fig. 4. Hydrolysis of the bound nucleotide. A subsection of the thermodynamic transition model during which the nucleotide is hydrolysed by the NBDs. "H" corresponds to the drug binding sites within the TMD in the inward facing high affinity configurations. "L" corresponds to the drug binding sites within the TMD in the outward facing low affinity configuration. The NBDs are in the engaged (N) conformations whilst drug is denoted by "d".

outward facing, low affinity conformation of the TMDs (L); in other words, the $^{d}HN^{ATP} \rightarrow {}^{d}LN^{ATP}$ transition shown in Fig. 3.

A question mark does hang over the conformation of the nucleotide occupancy and oligomerisation of P-gp NBDs at the ATP binding transition. According to the thermodynamic transition model (Fig. 4), the two possibilities for this transition are $^{d}HN^{ATP} \rightarrow {}^{d}LN^{ATP}$ or $^{d}Hn^{ATP} \rightarrow {}^{d}Ln^{ATP}$. That is, the alteration of the drug binding site from high (T) to low (R) affinity is either coerced by engaged NBDs (N) or disengaged NBDs (n). What is the evidence that NBD engagement is required for this alteration in drug affinity? As discussed above, the use of catalytic mutants of mouse P-gp (>85% identical to human P-gp) has supported the formation of a NBD engaged dimer containing tightly bound nucleotide (ATP) at a ratio of 1 mol/mol Pgp [16,20,31,52]. Intriguingly, formation of this occluded state is drug stimulated arguing for the binding of drug prior to the tight binding of nucleotide [31]. Thus, the transition $^{d}HN^{ATP} \rightarrow {}^{d}LN^{ATP}$ is the likely progression, rather than $^{d}Hn^{ATP} \rightarrow {}^{d}Ln^{ATP}$. In the thermodynamic transition model we suggest that when ATP is represented as the nucleotide, that this is the tightly occluded nucleotide described by Senior and co-workers, which is to undergo hydrolysis in subsequent steps (see Fig. 4).

A compelling argument can be made to discount the likelihood of the transition $^{d}Hn \rightarrow {}^{d}Ln$ as this would posit that the drug-binding site is switched spontaneously to a low affinity conformation, thereby producing translocation in the absence of nucleotide interaction. Were this scenario to occur, the energy obtained from nucleotide binding and hydrolysis is purely concerned with resetting the transporter following drug translocation. This is unlikely given the demonstrated interaction between ATP binding and drug binding [45,46].

One cautionary note should be discussed briefly. Although p[NH]ppA and ATPγS are frequently employed as non-hydrolysable analogues of ATP, the precise structures of the three nucleotides do differ. For example, p[NH]ppA has distinct bond lengths (P–N = 1.7 Å compared to P–O = 1.6 Å) and bond angles (P–N–P = 127° compared to P–O–P = 130°), as well as a lower pK_a for the terminal phosphate compared to ATP [53]. These stereochemical differences could manifest as non-identical effects of ATP compared to p[NH]ppA, as has

been seen in the F1-ATPase, where a 100-fold difference in apparent affinity for ATP and p[NH]ppA binding has been described [54,55]. However, in the case of P-gp such doubts are tempered by the observation that the potency for inhibition of ATPase activity by p[NH]ppA is the same as the Km for ATP hydrolysis [56]. A similar argument can be made to support the use of ATPγS.

4. Drug dissociation and ATP hydrolysis complete the initial stage of translocation

Evidence discussed in the previous section argues for the energy obtained from ATP binding (i.e., ΔG from the $^{d}Hn \rightarrow {}^{d}Hn^{ATP} \rightarrow {}^{d}HN^{ATP}$ transitions) as the initial impetus, or switch that facilitates drug translocation. The question remains as to whether the reduction in affinity of the drug binding sites to the $^{d}LN^{ATP}$ conformation correlates with a reorientation to the extracellular face of the membrane? This remains unresolved for P-gp. Fluorescence resonance energy transfer (FRET) measurements determined the distance between the donor fluorophore drug Hoechst 33342 and the acceptor fluorophore 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, which is speculated to bind exclusively to the Walker-A cysteine residues in the NBDs [57]. In the ATP free conformation the measured distance was 38 Å, and this was slightly reduced to 34 Å in the vanadate-trapped state of P-gp. Both values suggest that the bound Hoechst 33342 was close to the *cytoplasmic* side of the membrane, apparently incompatible with a drug binding site reorientation driven by ATP binding or hydrolysis [57]. Indeed this has been interpreted as a requirement for ADP release in the outward orientation of the drug binding site [57], though see below for a possible alternative interpretation. No higher resolution structural data exists for P-gp to address this issue. However, recent data for the *Salmonella typhimurium* MsbA demonstrated a partially engaged NBD dimer in a conformation believed to represent a post-hydrolytic state [14]. This structure contained bound substrate (lipopolysaccharide) flipped into the outer leaflet of the membrane [14]. In addition, data on the closely related bacterial ABC transporter LmrA, and the distantly related maltose transporter (detailed descriptions of which are beyond the scope of this review) clearly agree that the vanadate-trapped state has already reorientated the substrate (drug or maltose) binding site [9,58].

So, with the jury still out on the exact correlation between the low affinity drug binding site and the outward orientation, the assumption in the Fig. 4 is made that drug release (the $^{d}LN^{ATP} \rightarrow LN^{ATP}$ transition) occurs from an outward (extracellular) facing low affinity intermediate of the TMDs of P-gp. Once the drug-binding site has been converted to the low affinity conformation (L-conformation) the release, or dissociation, of drug is rapid and completes its translocation. So does the hydrolysis of ATP lead directly to the reacquisition of a high affinity inward facing conformation of the P-gp drug binding site(s)? It is clear from structural and numerous biochemical studies, that the hydrolysis of ATP causes significant conformational changes in the protein and that this transition intermediate (i.e., ADP · Pi bound) is distinct to the ATP bound form (outlined further in Table 1). However, radioligand binding studies argue against the proposal that ATP hydrolysis accompanies a return to the high affinity state since the $N^{ADP \cdot Pi}$ conformation (i.e., vanadate trapped) also displays low affinity binding of the transported substrate vinblastine, i.e., $LN^{ADP \cdot Pi}$, [46]. Further investigations are required to elucidate the molecular nature of the ADP · Pi bound intermediate of P-gp. One possibility is that ATP hydrolysis produces a reorientation of the drug-binding site to the inward facing conformation, but does not re-establish the high affinity drug binding site. This, indeed, would be compatible with the FRET data discussed above, in which the ADP · Vi state of P-gp had ligand apparently bound at the cytoplasmic face of the membrane [57].

As mentioned in the previous section, an alternative translocation sequence for P-gp proposes that ATP hydrolysis, rather than ATP binding, causes the switch in drug binding site(s) from high to low affinity. This proposal therefore implies that the $^{d}HN^{ATP} \rightarrow {}^{d}HN^{ADP \cdot Pi} \rightarrow {}^{d}LN^{ADP \cdot Pi} \rightarrow LN^{ADP \cdot Pi}$ transition sequence is employed. As a consequence, the ADP · Pi bound intermediate of P-gp ($^{d}HN^{ADP \cdot Pi}$) would retain bound drug and that dissociation would only occur following reorientation of this intermediate to the outward facing conformation $^{d}LN^{ADP \cdot Pi}$. However, this proposition cannot be reconciled if the ATP analogue bound, or vanadate trapped intermediates retain high affinity drug binding. This is clearly not the case for several P-gp substrates, although some notable exceptions have been reported. For example, studies with the non-transported P-gp modulator XR9576 (tariquidar) demonstrate that ATP binding does not confer a reduction in binding affinity [59]. This suggests that the communication between the NBDs and sites capable of drug translocation is different to the communication between the NBDs and sites of modulation [45]. This absence of binding site orientation may prevent the translocation of XR9576. A comparable observation was made for the photo-affinity prazosin derivative IAAP [35] and may reflect distinct lines of communication depending on either the site or the properties of the compound. Distinct lines of allosteric communication between the NBDs and different functionalities of the drug-interaction sites are also suggested by studies of the effect of labelling residues within TM α-helix 6 on drug stimulated ATPase activity [60]. Thus, the exact progression through the pharmacological cubic ternary model may be drug dependent and determine whether a drug is translocated or not.

5. Phosphate dissociation initiates resetting of the NBD and TMDs

As discussed in Sections 2 and 3, the precise stage of the translocation cycle at which the drug-binding site is reoriented and switches to a low affinity conformation remains the source of debate [35,45,46,61–63]. However, there is considerable agreement that immediately post ATP hydrolysis, P-gp displays lower affinity and reduced capacity for drug binding. This indicates that subsequent events in the catalytic cycle (i.e., release of phosphate and dissociation of ADP) mediate resetting of the transition intermediate $LN^{ADP \cdot Pi}$ to the starting conformation (Hn). Does ADP or inorganic phosphate dissociate first during the resetting phase shown in Fig. 5? The question was answered by the observation that vanadate completely inhibits the ATPase activity of P-gp by stably trapping the protein in a transition state containing bound Mg · ADP [7,35,45,46,52,61–63]. Based on structural

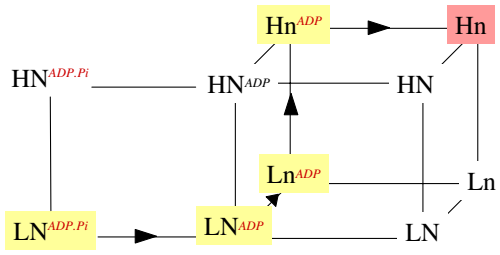

Fig. 5. Resetting phase of the translocation cycle. The subsection of the thermodynamic transition model that corresponds to resetting of P-gp for subsequent drug translocation events. "H" corresponds to the drug binding sites within the TMD in the inward facing high affinity configurations. "L" corresponds to the drug binding sites within the TMD in the outward facing low affinity configuration. The NBDs are in equilibrium between the separate (n) and engaged (N) conformations.

investigations with myosin, the pentacovalent vanadate ion is thought to form a complex with Mg · ADP to mimic the Mg · ADP · Pi transition intermediate [64]. Thus, the vanadate ion replaces the released Pi and strongly suggests that dissociation of Pi precedes that of ADP in the translocation pathway of P-gp.

The dissociation of phosphate is thought to provide the release of free energy derived from ATP hydrolysis. This hypothesis was validated by the observation that Pi only binds weakly to P-gp [7] and implies that the $LN^{ADP\cdot Pi} \rightarrow LN^{ADP}$ pathway proceeds avidly, with a large negative free energy change. That this occurs following dissociation of drug substrate confirms that the energy is used to reset the transporter. What is the released energy used for? The free energy released may be used directly to convert drug-binding sites from low affinity outward facing to the high affinity inward facing conformation (i.e., H → L). According to this scenario, the energy released from the dissociation of liberated phosphate destabilises the outward facing conformation of the TMDs (LN^{ADP}). This destabilised intermediate rapidly undergoes conformational change to the HN^{ADP} species. In turn, the NBDs disengage ($HN^{ADP} \rightarrow Hn^{ADP}$), thus enabling dissociation of the bound dinucleotide ($Hn^{ADP} \rightarrow Hn$) and the transporter is ready for a subsequent translocation event.

Alternatively, the energy may be used to disengage the NBDs from their closed conformation (i.e., N → n). Thus, phosphate dissociation from the NBDs ($LN^{ADP\cdot Pi} \rightarrow LN^{ADP}$) would lead to a rapid destabilisation of the NBD dimer and the disengagement of the monomers ($LN^{ADP} \rightarrow Ln^{ADP}$). Earlier in the translocation pathway it was the engagement of the NBDs that stimulated reorientation of the TMDs from the H to L-conformation. Conversely, it is tempting to view the disengagement of the NBDs as the stimulus for generating the reverse conformational change ($Ln^{ADP} \rightarrow Hn^{ADP}$). ADP readily dissociates from the Hn^{ADP} intermediate [31,52] and the transporter is reset to the basal conformation.

Urbatsch et al. [52] suggested that subsequent release of the bound Mg · ADP requires a slow monomerisation of the NBDs prior to the rapid dissociation of the dinucleotide. Ambudkar and co-workers [62] have suggested that the dissociation of Mg · ADP represents a rate-limiting step in the translocation pathway. These two observations also support the proposal that energy from Pi release is employed in the disengagement of the closed NBD configuration. The precise sequence of events in the resetting stage of the translocation pathway is likely to follow one of two routes described above. Whichever of the two routes is employed in the final stages of the translocation cycle of P-gp has yet to be elucidated. Pharmacological data again provides evidence that the Hn^{ADP} conformation exists: P-gp containing covalently attached ADP (i.e., post-release of Pi) has already been converted to the high affinity drug binding (H) conformation [46]. In addition, this intermediate could be covalently labelled with [^{32}P]-azido-ATP in the alternate NBD, suggesting that the NBDs exist in the disengaged, or open, conformation (Hn). However, the binding of ATP to the Hn^{ADP} conformation was not capable of affecting drug interaction; for instance, accelerating the $^{d}HN^{ATP} \rightarrow {}^{d}LN^{ATP}$ transition as observed in early stages of drug translocation. This indicates that the final step required to fully reset P-gp is dissociation of ADP. A corollary of this likely sequence of events is that the intermediates LN, HN and Ln are unlikely to be formed.

6. Summary

The sequence of events comprising the translocation pathway of P-gp outlined in this review (Fig. 1 and summary in Fig. 6) has been based on the considerable amount of available biochemical and pharmacological information. However, many key aspects remain unresolved including; the precise coupling stoichiometry between drug movement and ATP hydrolysis, and whether the route of coupling is via single or multiple routes from the drug binding sites. That there are multiple drug binding sites is well established, however, there is no detail on their molecular properties or how they discriminate interaction with substrates and modulators. Clearly, there is a need for further inquisition into the biochemistry of drug translocation and to provide high-resolution structural information in order to settle these issues. Once the biological enigma of multidrug translocation is fully understood we will be in a position to effectively develop (i) potent, selective P-gp inhibitors and/or (ii) anti-cancer drugs capable of evading the unwanted influence of P-gp.

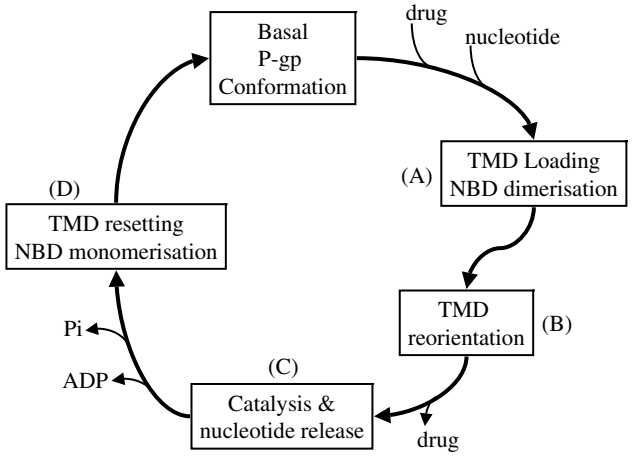

Fig. 6. Summary of the overall translocation process. The drug translocation process for P-gp comprises four distinct stages, (A) loading of P-gp with drug and nucleotide; (B) reorientation of the drug binding sites from high to low affinity; (C) nucleotide hydrolysis and (D) the resetting phase.

Acknowledgements: Research in the Callaghan laboratory was funded by a Cancer Research UK program grant (SP1861/0401). The authors thank the following for their discussion and scientific input over the course of the last decade: Catherine Martin, Janet Storm, Alice Rothnie, Mark Rosenberg and Christopher Higgins.

References

[1] Duraisingh, M.T. and Cowman, A.F. (2005) Contribution of the pfmdr1 gene to antimalarial drug-resistance. Acta Trop. 94, 181–190.
[2] Huang, Y. and Sadee, W. (2005) Membrane transporters and channels in chemoresistance and sensitivity of tumor cells. Cancer Lett.
[3] Langton, K.P., Henderson, P.J. and Herbert, R.B. (2005) Antibiotic resistance: multidrug efflux proteins, a common transport mechanism? Nat. Prod. Rep. 22, 439–451.
[4] van Veen, H.W. and Callaghan, R. (2003) in: ABC PROTEINS: From Bacteria to Man (Holland, I.B., Cole, S.P.C., Kuchler, K. and Higgins, C.F., Eds.), pp. 81–106, Elsevier, London.
[5] al-Shawi, M.K. and Senior, A.E. (1993) Characterization of the adenosine triphosphatase activity of Chinese hamster P-glycoprotein. J. Biol. Chem. 268, 4197–4206.
[6] Sarkadi, B., Price, E.M., Boucher, R.C., Germann, U.A. and Scarborough, G.A. (1992) Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. J. Biol. Chem. 267, 4854–4858.
[7] Urbatsch, I.L., Sankaran, B., Bhagat, S. and Senior, A.E. (1995) Both P-glycoprotein nucleotide-binding sites are catalytically active. J. Biol. Chem. 270, 26956–26961.
[8] Tombline, G., Bartholomew, L.A., Tyndall, G.A., Gimi, K., Urbatsch, I.L. and Senior, A.E. (2004) Properties of P-glycoprotein with mutations in the "catalytic carboxylate" glutamate residues. J. Biol. Chem. 279, 46518–46526.
[9] Chen, J., Sharma, S., Quiocho, F.A. and Davidson, A.L. (2001) Trapping the transition state of an ATP-binding cassette transporter: evidence for a concerted mechanism of maltose transport. Proc. Natl. Acad. Sci. USA 98, 1525–1530.
[10] Hopfner, K.P., Karcher, A., Shin, D.S., Craig, L., Arthur, L.M., Carney, J.P. and Tainer, J.A. (2000) Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. Cell 101, 789–800.
[11] Smith, P.C., Karpowich, N., Millen, L., Moody, J.E., Rosen, J., Thomas, P.J. and Hunt, J.F. (2002) ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer. Mol. Cell 10, 139–149.
[12] Chang, G. and Roth, C.B. (2001) Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. Science 293, 1793–1800.
[13] Chang, G. (2003) Structure of MsbA from *Vibrio cholera*: a multidrug resistance ABC transporter homolog in a closed conformation. J. Mol. Biol. 330, 419–430.
[14] Reyes, C.L. and Chang, G. (2005) Structure of the ABC transporter MsbA in complex with ADP · vanadate and lipopolysaccharide. Science 308, 1028–1031.
[15] Liu, R. and Sharom, F.J. (1997) Fluorescence studies on the nucleotide binding domains of the P-glycoprotein multidrug transporter. Biochemistry 36, 2836–2843.
[16] Urbatsch, I.L., Gimi, K., Wilke-Mounts, S. and Senior, A.E. (2000) Conserved walker A Ser residues in the catalytic sites of P-glycoprotein are critical for catalysis and involved primarily at the transition state step. J. Biol. Chem. 275, 25031–25038.
[17] Urbatsch, I.L., Beaudet, L., Carrier, I. and Gros, P. (1998) Mutations in either nucleotide-binding site of P-glycoprotein (Mdr3) prevent vanadate trapping of nucleotide at both sites. Biochemistry 37, 4592–4602.
[18] Lerner-Marmarosh, N., Gimi, K., Urbatsch, I.L., Gros, P. and Senior, A.E. (1999) Large scale purification of detergent-soluble P-glycoprotein from *Pichia pastoris* cells and characterization of nucleotide binding properties of wild-type, Walker A, and Walker B mutant proteins. J. Biol. Chem. 274, 34711–34718.
[19] Muller, M. et al. (1996) Altered drug-stimulated ATPase activity in mutants of the human multidrug resistance protein. J. Biol. Chem. 271, 1877–1883.
[20] Tombline, G., Bartholomew, L.A., Urbatsch, I.L. and Senior, A.E. (2004) Combined mutation of catalytic glutamate residues in the two nucleotide binding domains of P-glycoprotein generates a conformation that binds ATP and ADP tightly. J. Biol. Chem. 279, 31212–31220.
[21] Ramachandra, M., Ambudkar, S.V., Chen, D., Hrycyna, C.A., Dey, S., Gottesman, M.M. and Pastan, I. (1998) Human P-glycoprotein exhibits reduced affinity for substrates during a catalytic transition state. Biochemistry 37, 5010–5019.
[22] Shapiro, A.B. and Ling, V. (1995) Reconstitution of drug transport by purified P-glycoprotein. J. Biol. Chem. 270, 16167–16175.
[23] Taylor, A.M. et al. (2001) Detailed characterization of cysteine-less P-glycoprotein reveals subtle pharmacological differences in function from wild-type protein. Br. J. Pharmacol. 134, 1609–1618.
[24] Urbatsch, I.L., Al-Shawi, M.K. and Senior, A.E. (1994) Characterization of the ATPase activity of purified Chinese Hamster P-glycoprotein. Biochemistry 33, 7069–7076.
[25] Sharom, F.J. (1995) Characterization and functional reconstitution of the multidrug transporter. J. Bioenerg. Biomembr. 27, 15–22.
[26] Ambudkar, S.V., Cardarelli, C.O., Pashinsky, I. and Stein, W.D. (1997) Relation between the turnover number for vinblastine transport and for vinblastine-stimulated ATP hydrolysis by human P-glycoprotein. J. Biol. Chem. 272, 21160–21166.
[27] Eytan, G.D., Regev, R. and Assaraf, Y.G. (1996) Functional reconstitution of P-glycoprotein reveals an apparent near stoichiometric drug transport to ATP hydrolysis. J. Biol. Chem. 271, 3172–3178.
[28] Shapiro, A.B. and Ling, V. (1998) Stoichiometry of coupling of rhodamine 123 transport to ATP hydrolysis by P-glycoprotein. Eur. J. Biochem. 254, 189–193.
[29] Sharom, F.J., Yu, X. and Doige, C.A. (1993) Functional reconstitution of drug transport and ATPase activity in proteoliposomes containing partially purified P-glycoprotein. J. Biol. Chem. 268, 24197–24202.
[30] Delannoy, S., Urbatsch, I.L., Tombline, G., Senior, A.E. and Vogel, P.D. (2005) Nucleotide binding to the multidrug resistance P-glycoprotein as studied by ESR spectroscopy. Biochemistry 44, 14010–14019.
[31] Tombline, G., Muharemagic, A., White, L.B. and Senior, A.E. (2005) Involvement of the "occluded nucleotide conformation" of p-glycoprotein in the catalytic pathway. Biochemistry 44, 12879–12886.
[32] Jones, P.M. and George, A.M. (2004) The ABC transporter structure and mechanism: perspectives on recent research. Cell. Mol. Life Sci. 61, 1–18.
[33] Locher, K.P. (2004) Structure and mechanism of ABC transporters. Curr. Opin. Struct. Biol. 14, 426–431.
[34] Oloo, E.O. and Tieleman, D.P. (2004) Conformational transitions induced by the binding of MgATP to the vitamin B12 ATP-binding cassette (ABC) transporter BtuCD. J. Biol. Chem. 279, 45013–45019.
[35] Sauna, Z.E. and Ambudkar, S.V. (2000) Evidence for a requirement for ATP hydrolysis at two distinct steps during a single turnover of the catalytic cycle of human P-glycoprotein. Proc. Natl. Acad. Sci. USA 97, 2515–2520.
[36] Rothnie, A., Storm, J., Campbell, J., Linton, K.J., Kerr, I.D. and Callaghan, R. (2004) The topography of transmembrane segment six is altered during the catalytic cycle of P-glycoprotein. J. Biol. Chem. 279, 34913–34921.
[37] Sonveaux, N., Vigano, C., Shapiro, A.B., Ling, V. and Ruysschaert, J.M. (1999) Ligand-mediated tertiary structure changes of reconstituted P-glycoprotein. A tryptophan fluorescence quenching analysis. J. Biol. Chem. 274, 17649–17654.
[38] Julien, M. and Gros, P. (2000) Nucleotide-induced conformational changes in P-glycoprotein and in nucleotide binding site mutants monitored by trypsin sensitivity. Biochemistry 39, 4559–4568.

[39] Wang, G., Pincheira, R. and Zhang, J.-T. (1998) Dissection of drug-binding-induced conformational changes in P-glycoprotein. Eur. J. Biochem. 255, 383–390.
[40] Mechetner, E.B., Schott, B., Morse, B.S., Stein, W.D., Druley, T., Davis, K.A., Tsuruo, T. and Roninson, I.B. (1997) P-glycoprotein function involves conformational transitions detectable by differential immunoreactivity. Proc. Natl. Acad. Sci. USA 94, 12908–12913.
[41] Nagy, H., Goda, K., Arceci, R., Cianfriglia, M., Mechetner, E. and Szabo Jr., G. (2001) P-glycoprotein conformational changes detected by antibody competition. Eur. J. Biochem. 268, 2416–2420.
[42] Rosenberg, M.F., Callaghan, R., Modok, S., Higgins, C.F. and Ford, R.C. (2005) Three-dimensional structure of P-glycoprotein: the transmembrane regions adopt an asymmetric configuration in the nucleotide-bound state. J. Biol. Chem. 280, 2857–2862.
[43] Rosenberg, M.F., Kamis, A.B., Callaghan, R., Higgins, C.F. and Ford, R.C. (2003) Three-dimensional structures of the mammalian multidrug resistance P-glycoprotein demonstrate major conformational changes in the transmembrane domains upon nucleotide binding. J. Biol. Chem. 278, 8294–8299.
[44] Rosenberg, M.F. et al. (2001) Repacking of the transmembrane domains of P-glycoprotein during the transport ATPase cycle. EMBO J. 20, 5615–5625.
[45] Martin, C., Berridge, G., Mistry, P., Higgins, C., Charlton, P. and Callaghan, R. (2000) Drug binding sites on P-glycoprotein are altered by ATP binding prior to nucleotide hydrolysis. Biochemistry 39, 11901–11906.
[46] Martin, C., Higgins, C.F. and Callaghan, R. (2001) The vinblastine binding site adopts high- and low-affinity conformations during a transport cycle of P-glycoprotein. Biochemistry 40, 15733–15742.
[47] Stenham, D.R., Campbell, J.D., Sansom, M.S., Higgins, C.F., Kerr, I.D. and Linton, K.J. (2003) An atomic detail model for the human ATP binding cassette transporter P-glycoprotein derived from disulfide cross-linking and homology modeling. FASEB J. 17, 2287–2289.
[48] Loo, T.W. and Clarke, D.M. (1997) Drug-stimulated ATPase activity of human P-glycoprotein requires movement between transmembrane segments 6 and 12. J. Biol. Chem. 272, 20986–20989.
[49] Loo, T.W. and Clarke, D.M. (2001) Determining the dimensions of the drug-binding domain of human P-glycoprotein using thiol cross-linking compounds as molecular rulers. J. Biol. Chem. 276, 36877–36880.
[50] Loo, T.W. and Clarke, D.M. (2002) Vanadate trapping of nucleotide at the ATP-binding sites of human multidrug resistance P-glycoprotein exposes different residues to the drug-binding site. Proc. Natl. Acad. Sci. USA 99, 3511–3516.
[51] Liu, R. and Sharom, F.J. (1996) Site-directed fluorescence labeling of P-glycoprotein on cysteine residues in the nucleotide binding domains. Biochemistry 35, 11865–11873.
[52] Urbatsch, I.L., Tyndall, G.A., Tombline, G. and Senior, A.E. (2003) P-glycoprotein catalytic mechanism: studies of the ADP-vanadate inhibited state. J. Biol. Chem. 278, 23171–23179.
[53] Yount, R.G. (1975) ATP analogs. Adv. Enzymol. Relat. Areas Mol. Biol. 43, 1–56.
[54] Cross, R.L. and Nalin, C.M. (1982) Adenine nucleotide binding sites on beef heart F1-ATPase. Evidence for three exchangeable sites that are distinct from three noncatalytic sites. J. Biol. Chem. 257, 2874–2881.
[55] Grubmeyer, C., Cross, R.L. and Penefsky, H.S. (1982) Mechanism of ATP hydrolysis by beef heart mitochondrial ATPase. Rate constants for elementary steps in catalysis at a single site. J. Biol. Chem. 257, 12092–12100.
[56] al-Shawi, M.K., Urbatsch, I.L. and Senior, A.E. (1994) Covalent inhibitors of P-glycoprotein ATPase activity. J. Biol. Chem. 269, 8986–8992.
[57] Qu, Q. and Sharom, F.J. (2002) Proximity of bound Hoechst 33342 to the ATPase catalytic sites places the drug binding site of P-glycoprotein within the cytoplasmic membrane leaflet. Biochemistry 41, 4744–4752.
[58] van Veen, H.W., Margolles, A., Muller, M., Higgins, C.F. and Konings, W.N. (2000) The homodimeric ATP-binding cassette transporter LmrA mediates multidrug transport by an alternating (two-cylinder engine) mechanism. EMBO J. 19, 2503–2514.
[59] Martin, C., Berridge, G., Higgins, C.F., Mistry, P., Charlton, P. and Callaghan, R. (2000) Communication between multiple drug binding sites on P-glycoprotein. Mol. Pharmacol. 58, 624–632.
[60] Rothnie, A., Storm, J., McMahon, R., Taylor, A., Kerr, I.D. and Callaghan, R. (2005) The coupling mechanism of P-glycoprotein involves residue L339 in the sixth membrane spanning segment. FEBS Lett. 579, 3984–3990.
[61] Al-Shawi, M.K., Polar, M.K., Omote, H. and Figler, R.A. (2003) Transition state analysis of the coupling of drug transport to ATP hydrolysis by P-glycoprotein. J. Biol. Chem. 278, 52629–52640.
[62] Kerr, K.M., Sauna, Z.E. and Ambudkar, S.V. (2001) Correlation between steady-state ATP hydrolysis and vanadate-induced ADP trapping in human P-glycoprotein. J. Biol. Chem. 276, 8657–8664.
[63] Sauna, Z.E. and Ambudkar, S.V. (2001) Characterization of the catalytic cycle of ATP hydrolysis by human P-glycoprotein. The two ATP hydrolysis events in a single catalytic cycle are kinetically similar but affect different functional outcomes. J. Biol. Chem. 276, 11653–11661.
[64] Mildvan, A.S. (1997) Mechanisms of signaling and related enzymes. Proteins 29, 401–416.
[65] Sonveaux, N., Shapiro, A.B., Goormaghtigh, E., Ling, V. and Ruysschaert, J.M. (1996) Secondary and tertiary structure changes of reconstituted P-glycoprotein. A Fourier transform attenuated total reflection infrared spectroscopy analysis. J. Biol. Chem. 271, 24617–24624.
[66] Wang, G., Pincheira, R., Zhang, M. and Zhang, J.T. (1997) Conformational changes of P-glycoprotein by nucleotide binding. Biochem. J. 328 (Pt 3), 897–904.
[67] Druley, T.E., Stein, W.D. and Roninson, I.B. (2001) Analysis of MDR1 P-glycoprotein conformational changes in permeabilized cells using differential immunoreactivity. Biochemistry 40, 4312–4322.