Minireview

The catalytic cycle of P-glycoprotein

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Abstract P-glycoprotein is a plasma-membrane glycoprotein which confers multidrug-resistance on cells and displays ATP-driven drug-pumping in vitro. It contains two nucleotide-binding domains, and its structure places it in the 'ABC transporter' family. We review recent evidence that both nucleotide-sites bind and hydrolyse Mg-ATP. The two catalytic sites interact strongly. A minimal scheme for the MgATP hydrolysis reaction is presented. An alternating catalytic sites scheme is proposed, in which drug transport is coupled to relaxation of a high-energy catalytic site conformation generated by the hydrolysis step. Other ABC transporters may show similar catalytic features.

Key words: Multidrug-resistance; P-glycoprotein; ATPase catalytic cycle

1. Introduction

P-Glycoprotein (Pgp) (also called multidrug-resistance protein) is a plasma-membrane glycoprotein which confers multidrug-resistance (MDR) phenotype on cells [1–4]. It is of considerable interest because it provides one mechanism of resistance to chemotherapy in cancer [5–7] and is also a prominent member of the 'ABC transporter' family of membrane proteins [6,8].

Earlier work on Pgp utilized cell and molecular biology methodologies to describe its structure and function. Recently sufficient experimental material has been obtained, in the form of Pgp-enriched membrane preparations and purified Pgp, to facilitate biochemical investigations, which have revealed details of catalytic, ATP hydrolysis properties. This review summarises recent work on catalysis, with the hope that it will stimulate further understanding of Pgp and ABC transporters generally.

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Abbreviations: Pgp, P-glycoprotein; MDR, multidrug-resistance; TMD, trans-membrane domain; NBS, nucleotide-binding site; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; Vi, orthovanadate; DCCD, dicyclohexyl-carbodiimide, FITC, fluorescein isothiocyanate.

2. Indications that Pgp hydrolyses ATP

Early work which established that Pgp acts by excluding cytotoxic drugs from cells suggested also that it utilizes energy from ATP [9,10], and subsequent reports confirmed this. Recent papers [11–16] present evidence for ATP-driven drug-pumping by Pgp in vitro, with non-hydrolysable ATP analogs being ineffective.

The amino acid sequence suggests Pgp might be an ATPase. Pgp is a single polypeptide of ~1280 residues with typical ABC transporter structure profile, conveniently represented as {TMD1-NBS1-TMD2-NBS2}, where TMD stands for 'trans-membrane domain' (containing six trans-membrane α-helices) and NBS stands for 'nucleotide-binding site'. Each NBS contains 'Homology A' and 'Homology B' consensus sequences [17], diagnostic of nucleotide-binding and common in ATPase enzymes. NBS1 and NBS2 are strongly homologous to each other, with ~66% identical residues in human and rodent Pgp.

These indications stimulated studies to establish whether membrane-bound and purified Pgp catalyse ATP hydrolysis.

3. Demonstration and characterization of ATPase activity

A crude membrane fraction from baculovirus-infected Sf9 cells showed considerable ATPase activity when the virus carried human MDR1 (Pgp) cDNA, but not with 'sham' virus [18]. ATPase activity was stimulated by verapamil, a known 'modulator' or 'chemo-sensitizer' of Pgp-mediated MDR phenotype, and also by the drugs vincristine, vinblastine, and daunomycin, to which Pgp confers resistance in cancer cells. Al-Shawi and Senior [19] obtained purified plasma membranes which were highly-enriched in Pgp from multidrug-resistant Chinese hamster ovary cells. These membranes showed substantial ATPase activity, referable to Pgp, which was stimulated by verapamil and a range of other drugs.

Chinese hamster Pgp was solubilized in detergent, purified to homogeneity, and reconstituted in proteoliposomes [20,21]. The pure reconstituted Pgp showed substantial drug-stimulated ATPase activity. Human Pgp was also purified to homogeneity and reconstituted in proteoliposomes with retention of activity [22], and active, purified, soluble Chinese hamster Pgp in the form of lipid–protein aggregates was described [23].

Membrane-bound and purified Pgp preparations show similar enzymatic properties. (Pgp occurs as three isoforms in rodents and two in human. Work on ATPase of Chinese hamster Pgp is referable to isoform-1 (Pgp1), the isoform overexpressed in the cell lines used [24,25]. Studies on ATPase of human Pgp used MDR1 isoform which confers MDR and is equivalent to Chinese hamster Pgp1. No significant differences between human MDR1 and Chinese hamster Pgp1 have been seen.)

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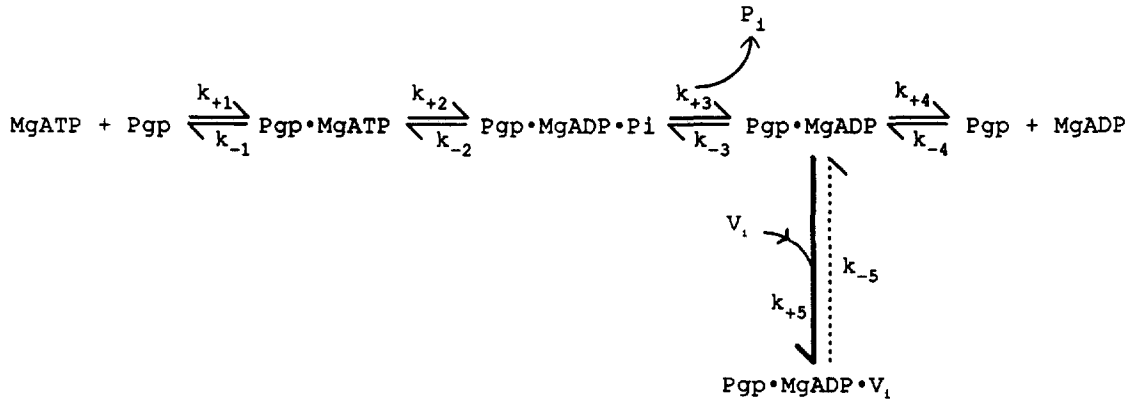

Fig. 1. Scheme for vanadate-inhibition of P-glycoprotein ATPase activity and ATP hydrolysis.

Maximal, drug-stimulated Mg-ATP hydrolysis turnover rate = 10–20 s^{-1}, K_m(Mg-ATP) = ~1 mM, k_{cat}/K_m(Mg-ATP) = ~10^4 $M^{-1} \cdot s^{-1}$. Only a single K_m(Mg-ATP) is apparent, which does not materially change in presence of drugs. Mg-ADP and Mg-AMPP-NP are competitive inhibitors with K_i values 0.35–0.70 mM. A diverse range of nucleoside triphosphates is hydrolysed. Overall we conclude that Mg-ATP is the physiological substrate and it is hydrolysed at a catalytic site or sites which have low affinity and low specificity for nucleotides [26].

Pgp preparations (membranes or purified) show significant 'basal' ATPase activity in absence of drug, the origin of which is not yet understood. It might be due to activation by endogenous transport site ligand (lipid?). The degree of enhancement of ATPase by drugs varies among preparations. With verapamil, which elicits maximal turnover, the degree of stimulation above basal ranges from 2.5- to 11-fold. It has been established that the lipid milieu affects both basal activity and degree of stimulation by drugs [23,27,28].

Degree of glycosylation does not appear to affect catalysis. Human Pgp expressed in Sf9 or human cell-lines (above) showed similar ATPase characteristics. It may be noted that Pgp confers drug-resistance in yeast and *Escherichia coli* [29–31]. The role of phosphorylation in modulating Pgp activity remains unclear. Mutational analyses failed to detect a critical role for phosphorylation sites [32]. A recent report suggested that phosphorylation increases ATPase activity of Pgp in membranes by ~2-fold [33]. Calmodulin was shown to modulate drug transport by Pgp in membrane vesicles [34], and may significantly affect ATPase activity. It is apparent therefore that a number of identified entities, including lipid environment, phosphorylation and calmodulin may modulate Pgp ATPase activity, although their physiological roles have yet to be defined.

Several potent inhibitors of Pgp ATPase activity have been identified (Table 1) and have been valuable in characterisation. Also listed in Table 1 are compounds known to potently inhibit other membrane ATPases, which do not markedly inhibit Pgp, and hence are useful tools for distinguishing Pgp activity.

Mutations affect ATPase activity significantly. Several mutations located in the TMD were shown to affect basal and drug-stimulated ATPase activity of purified, reconstituted human Pgp [22]. These included F335A, which gave considerable increase in ATPase, and F978A which gave large decrease. G141V, G185V and G830V caused relative changes in degree of activation of ATPase by vinblastine, verapamil and colchicine. Rao [35] studied the G185V mutation in human Pgp using the membrane fraction from Sf9 cells, and obtained similar results. It was apparent from these studies that functional communication between the TMD and catalytic sites was retained, and that relative effects of mutations on ATPase activity paralleled those on drug-exclusion seen in whole cells. Mutations occurring within the NBS will be discussed later.

4. Evidence that both predicted nucleotide-binding sites bind Mg-ATP

NEM inactivates Pgp ATPase potently and reacts covalently at two sites. Mg-ATP protects against inactivation and reaction. Labeling with radioactive NEM occurred equally in N- and C-terminal halves of the molecule [21,25]. (Assay of distribution of label between NBS1 and NBS2 is facilitated by mild trypsin digestion, which splits Pgp into N- and C-terminal 'halves' [36].) From these findings it was postulated that NEM reacts with two specific Cys residues, located in the Homology A sequences of NBS1 and NBS2 [25]. The Homology A sequence in NBS1 is GNSGCGKS and that in NBS2 is GSSGCGKS. Loo and Clarke [37] proved that the highlighted

Table 1
Inhibition of ATPase activity of P-glycoprotein

Compound	EC_{50}[a,b]
Orthovanadate (Vi)	10 μM
N-ethylmaleimide (NEM)	5 μM
NBD-Cl	10 μM
Bafilomycin A_1	1 μM[c]
Fluoroaluminate	60 μM
EGTA	>1 mM
Ouabain	>10 mM
DCCD	0.2 mM
NaN_3	>50 mM
FITC	0.2 mM

[a]EC_{50} = concentration for 50% inhibition.
[b]Except as noted, values are those measured in our laboratory with plasma-membrane or purified reconstituted Chinese hamster Pgp [19,21,25]. Average numbers are presented.
[c]Data for purified Pgp from [23]. Concanamycin A and B inhibited with similar potency. Bafilomycin A_1 inhibited membrane Pgp by 50% at 10 μM.

Cys residues were the ones that reacted with NEM. They generated Cys-free Pgp (which retained normal activity in regard to MDR phenotype and ATPase) then re-inserted the Homology A Cys residues separately. Each of the two resultant mutant Pgp molecules showed NEM-sensitive ATPase, and Mg-ATP protected with similar efficacy in each case. These data show that MgATP binds to both NBS1 and NBS2, with approximately equal affinity.

Mg-8-azido-ATP is a UV-photoactivated analog of Mg-ATP, often used to label Mg-ATP binding sites of proteins. It is a good hydrolysis substrate for Pgp, with K_m and k_{cat}/K_m values similar to Mg-ATP [21,25]. At saturating concentrations, Mg-8-azido-ATP was seen to label Pgp with stoichiometry approaching 2 mol/mol [25]. The label was equally distributed in NBS1 and NBS2 and labeling was protected by MgATP. These results show that both NBS1 and NBS2 bind Mg-8-azido-ATP and Mg-ATP.

Loo and Clarke [38] constructed 'half-molecules' of Pgp corresponding to {TMD1 plus NBS1} and {TMD2 plus NBS2}, respectively, and expressed them in Sf9 cells. Both showed ATPase activity, indicating that both NBS1 and NBS2 have the potential capability to bind and hydrolyse ATP. Basal ATPase was similar in both half-molecules, but there was no stimulation by drugs except when they were co-expressed. Thus, interaction between the two half-molecules was critical for normal function.

5. Evidence that the two nucleotide-binding sites interact

Work was described above in which mutant Pgp proteins containing a single Cys located in either the Homology A sequence of NBS1 or NBS2 were constructed [37]. Reaction of either Cys with NEM was seen to eliminate all ATPase activity, showing that inactivated NBS1 blocked hydrolysis of Mg-ATP in NBS2 or vice versa. This argues strongly that the two NBS interact.

NBD-Cl inactivated Pgp ATPase and reacted covalently with stoichiometry of 1.1 mol/mol. Inactivation and labeling were protected by Mg-ATP [19] and covalent incorporation of NBD-Cl occurred predominantly in NBS2 [21]. Since reaction of NBD-Cl in NBS2 eliminated all ATPase activity, inactivated NBS2 must interact with NBS1 to block Mg-ATP hydrolysis in that site.

Azzaria et al. [39] introduced mutations (Gly→Ala or Lys→Arg) into the Homology A sequences of either NBS1 or NBS2. Each mutation was sufficient to block drug-transport by Pgp in cells. Thus it was apparent that both nucleotide sites must be intact for normal function. Loo and Clarke [37] extended this approach, showing that the mutations K433M, K1076M, G432S and G1075S in the Homology A sequences of either NBS1 or NBS2 in human Pgp abolish drug-exclusion capability in cells and eliminate ATPase activity in membranes. This argues strongly that the two catalytic sites must interact, and coincidentally demonstrates an obligatory link between ATPase and drug-exclusion activity.

Persuasive data showing interaction between nucleotide sites has come from vanadate-trapping experiments. It was found that orthovanadate (Vi) inhibits Pgp ATPase by trapping nucleotide in a catalytic site [40]. Whether Mg-ATP or Mg-ADP was initially preincubated with Pgp and Vi, the resultant trapped nucleotide was ADP, showing that in the case of Mg-ATP, at least one turnover occurred before onset of inhibition. Reactivation of vanadate-inhibited Pgp was slow, and correlated well with release of trapped nucleotide. Significantly, vanadate-trapping of ADP at just one catalytic site per Pgp was sufficient to block all ATPase and release of the single trapped ADP regenerated ATPase activity fully.

Mg-8-azido-ATP substituted well for Mg-ATP in vanadate-trapping experiments, and allowed determination of which NBS was the one at which trapping occurred [41]. The procedure followed was to incubate radioactive Mg-8-azido-ATP with Pgp and Vi, separate inhibited Pgp from free Vi and nucleotide, subject it to UV-photoactivation, and determine distribution of label between N- and C-halves of the protein. The label was distributed equally, showing that vanadate-trapping occurred non-selectively in both NBS1 and NBS2. This experiment shows that both nucleotide-sites hydrolyze 8-azido-ATP and that trapping of nucleotide at either catalytic site completely blocks hydrolysis at both sites. The two sites have approximately the same apparent affinity for 8-azido-ATP, consistent with the fact that there is only a single apparent K_m for Mg-8-azido-ATP or MgATP [21,25].

6. The ATP hydrolysis reaction

We have not detected any covalent Pgp-phosphate catalytic intermediate species and have concluded that the ATPase reaction does not involve one. Rather, experiments described above suggested the scheme for vanadate-inhibition shown in Fig. 1, from which a minimal scheme for Mg-ATP hydrolysis emerges. The major findings were as follows. First, incubation of Pgp with Vi and either Mg-ATP or Mg-ADP induces inhibition but the trapped nucleotide is ADP in both cases. Second, Vi competes with P_i but not with Mg-ATP or Mg-ADP. P_i is a weakly-binding ligand with K_i = ~100 mM. Third, vanadate-inhibition occurs rapidly with Mg-ATP but slowly with Mg-ADP. Fourth, P_i is a weak, mixed-type inhibitor of ATPase activity [41].

We propose that vanadate-inhibition occurs by steps $(k_{+1}\cdot k_{+2}\cdot k_{+3}\cdot k_{+5})$ (with MgATP) or by steps $(k_{-4}\cdot k_{+5})$ (with MgADP), and that ATP hydrolysis occurs by steps $(k_{+1}\cdot k_{+2}\cdot k_{+3}\cdot k_{+4})$ (see Fig. 1). Since reactivation of vanadate-inhibited Pgp is slow ($k_{app} = 1.4\times10^{-4}\ s^{-1}$ at 37°C) and overall ATPase turnover is ~10–20 s^{-1} at 37°C, step k_{-5} is rate-limiting in reactivation. Given the chemical analogy between Vi and P_i, there are strong reasons to think that Vi in the Pgp·Mg-ADP·Vi complex occupies the same position as P_i derived from MgATP during hydrolysis, and that the Pgp·Mg-ADP·Vi complex resembles the transition state conformation of the normal reaction pathway.

7. Alternation of the two catalytic sites in catalysis

The finding that vanadate-trapping of nucleotide at either catalytic site blocks ATP hydrolysis at both sites suggests that when one site enters the transition state conformation the other cannot do so, and implies that the sites may undergo ATP hydrolysis alternately. Mutations or chemical modifications which inactivate one catalytic site also block catalysis at the other, intact site. In these cases, if alternation of sites is required, then catalysis would halt after a single turnover in the intact site.

Fig. 2 depicts a postulated alternating catalytic sites cycle,

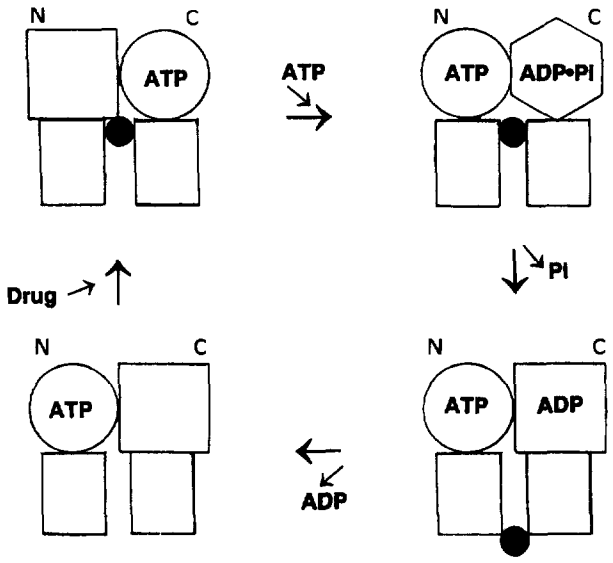

Fig. 2. Postulated alternating catalytic sites cycle of ATP hydrolysis by P-glycoprotein. Rectangles, represent the two Pgp TMD (trans-membrane domains); Circles, squares, and hexagon, represent different conformations of the N- and C-catalytic sites (NBS1 and NBS2, respectively). (Top left) The N-catalytic site is empty, the C-catalytic site has bound ATP, drug is bound at inside-facing transport site. (Top right) We suggest that ATP binding at the N-site allows ATP hydrolysis at the C-site, inducing a conformation at the C-site which prohibits hydrolysis at the N-site. The conformation at the C-site immediately after bond-cleavage is a high chemical potential state with bound ADP·Pi, shown as a *hexagon*. (Bottom right) Relaxation of the C-site conformation occurs, coupled to drug movement from inside-facing, higher-affinity to outside-facing, lower-affinity, and P_i is released. (Bottom left) Drug and ADP dissociate. Drug binds at the inner side and in *top left* N- and C-sites have now reversed their relationship. In the next cycle, ATP hydrolysis will occur in the N-site.

which incorporates also a proposal for coupling of drug transport to ATP hydrolysis. Pgp differs from other transport ATPases in that it shows no high-affinity binding site for Mg-ATP, nor does it utilize a covalent E~P catalytic intermediate. Changes in free energy associated with such species during the catalytic cycle are thought to be coupled to conformational changes at transport substrate binding sites in other transport ATPases [42]. In Pgp, P_i binding occurs with relatively weak affinity (above), implying that a large free energy change occurs during catalysis before the stage of P_i release. We postulate therefore that the ATP hydrolysis step itself generates a Pgp·Mg-ADP·P_i conformation of high chemical potential. Relaxation of this conformation, probably through intermediates, could be coupled to movement of a drug-binding site from inside-facing aspect of higher affinity to outside-facing aspect of lower affinity.

8. Conclusions

(1) Pgp is an ATPase and ATPase activity is obligatorily linked to conferral of MDR phenotype in cells. Both predicted nucleotide-binding sites bind Mg-ATP with approximately the same affinity, and both hydrolyze Mg-ATP. The two catalytic sites interact strongly and cannot hydrolyze Mg-ATP independently. A minimal reaction scheme for ATP hydrolysis is presented, and an alternating catalytic sites cycle is proposed. (2) Understanding coupling of ATP hydrolysis to drug transport is a major goal. We postulate that drug transport is coupled to relaxation of a high energy catalytic site conformation which is generated by the hydrolysis step. (3) It is not unreasonable to speculate that several, perhaps most, of the ABC transporters have nucleotide-binding and ATP hydrolysis characteristics similar to those of Pgp. The designation of the ABC transporter family is based on similarity of predicted structure; it is important to find out whether there are also basic functional similarities. The experimental approaches reviewed here may prove valuable in this regard. (4) Work on Pgp underscores the concept that transport ATPases gain a key advantage from the use of multiple, highly-cooperative catalytic sites reacting sequentially. What the key advantage is, however, remains to be clarified.

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