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The Mechanism of Action of Multidrug-Resistance-Linked P-Glycoprotein

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P-glycoprotein (Pgp), the ATP-binding cassette (ABC) transporter, confers multidrug resistance to cancer cells by extruding cytotoxic natural product amphipathic drugs using the energy of ATP hydrolysis. Our studies are directed toward understanding the mechanism of action of Pgp and recent work deals with the assessment of interaction between substrate and ATP sites and elucidation of the catalytic cycle of ATP hydrolysis. The kinetic analyses of ATP hydrolysis by reconstituted purified Pgp suggest that ADP release is the rate-limiting step in the catalytic cycle and the substrates exert their effect by modulating ADP release. In addition, we provide evidence for two distinct roles for ATP hydrolysis in a single turnover of Pgp, one in the transport of drug and the other in effecting conformational changes so as to reset the transporter for the next catalytic cycle. Detailed kinetic measurements determined that both nucleotide-binding domains behave symmetrically and during individual hydrolysis events the ATP sites are recruited in a random manner. Furthermore, only one nucleotide site hydrolyzes ATP at any given time, causing (in this site) a conformational change that drastically decreases (>30-fold) the affinity of the second site for ATP-binding. Thus, the blocking of ATP-binding to the second site while the first one is in catalytic conformation appears to be the basis for the alternate catalytic cycle of ATP hydrolysis by Pgp, and this may be applicable as well to other ABC transporters linked with the development of multidrug resistance.

KEY WORDS: ABC transporter; ATP hydrolysis; cancer chemotherapy; catalytic cycle; multidrug resistance; P-glycoprotein.

MULTIDRUG RESISTANCE AND THE ABC TRANSPORTERS

The resistance of cancer cells to cytostatic agents has been a significant impediment to the effective chemotherapy of cancer. Cancer cells grown in vitro and selected for resistance to specific anticancer drugs allowed two major classes of drug-resistant cells to be recognized: cells resistant to a single class of drugs with the same mechanism of action and cells resistant to chemically diverse drugs with multiple mechanisms of action (Endicott and Ling, 1989; Gottesman and Pastan, 1993). The latter phenomenon was called multiple (or multi) drug resistance (MDR).[3] Although numerous factors have been implicated in the development of MDR, a large body of evidence strongly supports an important role for energy-dependent pump systems that either exclude or extrude chemotherapeutic agents from cells (for reviews see Ambudkar *et al.*, 1999; Borst, 1999; Gottesman and Pastan, 1993; Sharom, 1997). Thus, decreased influx and/or increased efflux of drugs from cells, resulting in reduced accumulation of drugs in cells, is responsible for drug resistance. This is achieved by the overexpression of energy-dependent efflux systems.

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[3] Key to abbreviations: ABC, ATP-binding cassette; AMPPNP, 5′-adenylylimididiphosphate; DMSO, dimethyl sulfoxide; [^{125}I]IAAP, [^{125}I]iodoarylazidoprazosin; MDR, multidrug resistance; PAGE, polyacrylamide gel electrophoresis; Pgp, P-glycoprotein; Vi, vanadate.

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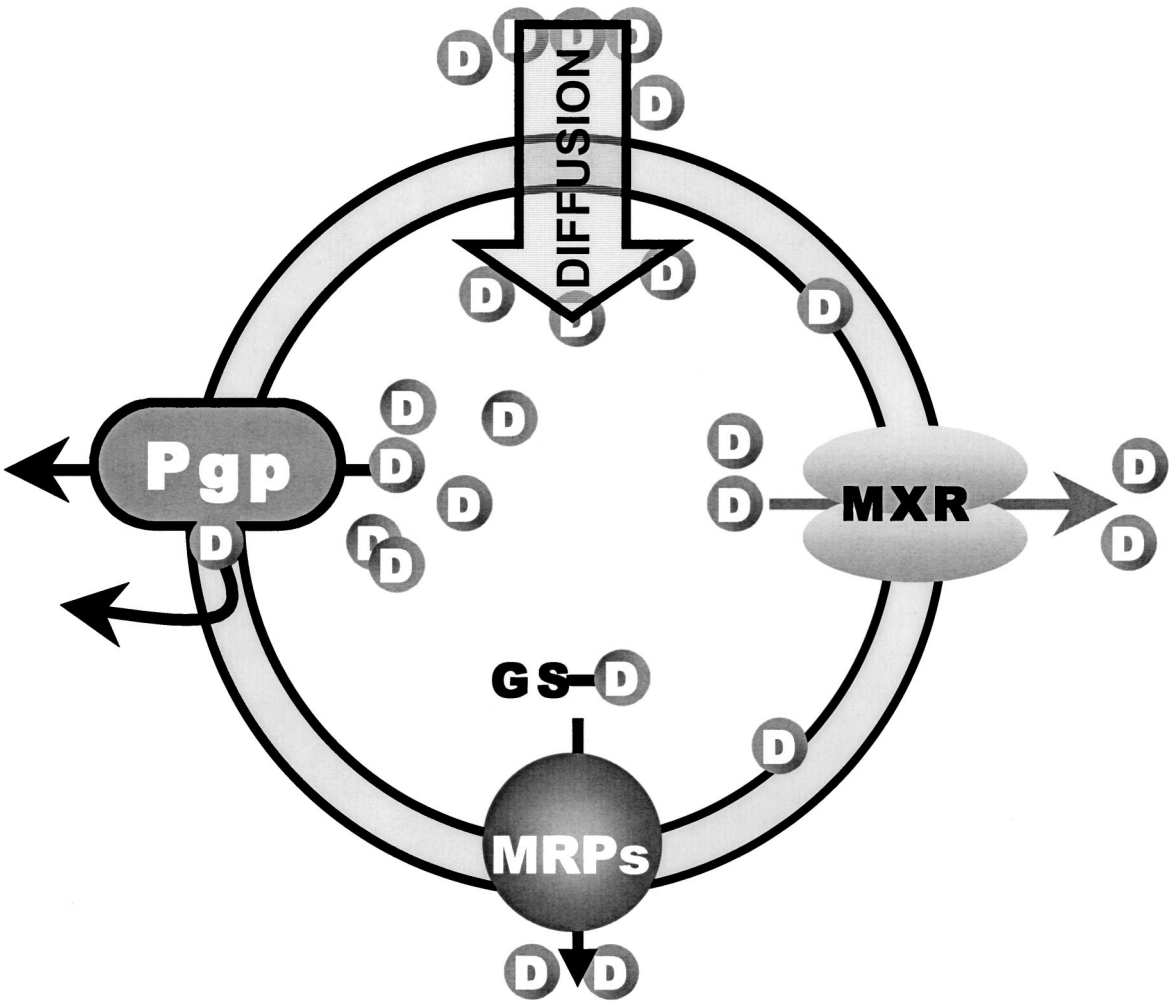

Fig. 1. Role of ABC transporters in the development of the MDR phenotype in cancer cells. Cancer cells show resistance to cytotoxic agents via one or more of several mechanisms. Most natural product hydrophobic drugs (D) enter the cell by diffusion. These may be pumped out by Pgp using the energy of ATP hydrolysis, either from the cytoplasm or from the membrane phase itself before they reach the cytoplasm. Drugs complexed with glutathione (GSH) may also be transported out of cells by MRPs (MRP1–4). The "half-transporter" MXR (or ABCP or BCRP) also effluxes drugs in an energy-dependent manner, with the dimer possibly being the functional unit. Besides these ATP-dependent transport systems, the cells may also acquire resistance by a number of intracellular mechanisms such as intracellular compartmentalization, metabolic degradation, altered cell cycle, and increased DNA repair (Gottesman *et al.*, 1994).

The human *MDR1* gene product P-glycoprotein (Pgp) was the first ATP-dependent system discovered that was implicated in MDR (Juliano and Ling, 1976) and has been extensively characterized (different aspects of Pgp are reviewed in (Ambudkar *et al.*, 1999; Gottesman and Pastan, 1993; Schinkel, 1997; Senior *et al.*, 1995a,b; Sharom, 1997). However, the overexpression of Pgp is not the only cause of MDR. Figure 1 illustrates the transport proteins implicated in MDR. Many cells selected for resistance do not show increased levels of Pgp but nonetheless are resistant to a broad range of natural product drugs (Cole and Deeley, 1998; Loe *et al.*, 1996). Another member of the ATP-binding cassette (ABC) superfamily, the MDR associated protein 1 (MRP1), is expressed in some of these cell lines at elevated levels (Cole *et al.*, 1992). MRP1 is similar to Pgp in that it is capable of decreasing intracellular levels of drugs, and is ATP-dependent. In addition, multidrug-resistant tumor cell lines that lack overexpression of both Pgp and MRP have been described (Doyle *et al.*, 1998; Miyake *et al.*, 1999). Several of these were selected in mitoxantrone and recently, the mitoxantrone resistance (*MXR*) associated gene has been cloned and characterized (Allikmets *et al.*, 1998; Doyle *et al.*, 1998; Miyake *et al.*, 1999). The *MXR* gene product (also known as BCRP, breast cancer resistance protein or ABCP) is a 655 amino acid protein and constitutes a "half-transporter"

(see below). Although all three transporters are members of the ABC superfamily of transport proteins, they belong to different subgroups (see Dean and Allikmets, 2001). Hydropathy plots of these proteins have led to the secondary structure models. Pgp consists of two putative transmembrane domains, each consisting of six hydrophobic transmembrane helices and one ATP-binding domain. The carboxy terminal half is 43% homologous to the amino terminal half (Chen *et al.*, 1986). While Pgp has two membrane spanning domains, MRP1 has three (Tusnady *et al.*, 1997); however the first domain does not appear to be necessary for function (Bakos *et al.*, 1998). Similarly the MXR protein, although a "half-transporter" manifests the basic unit of the ABC transporter, i.e. a membrane spanning domain, predicted to have six transmembrane helices and a nucleotide-binding site (Miyake *et al.*, 1999). As both halves of Pgp have been shown to be necessary for function (Loo and Clarke, 1995a,b) it is assumed that the functional unit of MXR would be a dimer. Finally, although Pgp, MRP1, and MXR show fundamental similarities in their architecture, they show both an overlap and specificity vis-à-vis the drug-substrates they can extrude (Litman *et al.*, 2000). It is clear that the resistance of tumor cells to cytotoxic chemotherapeutic drugs is a major clinical problem and the pharmacological reversal of Pgp has been a major focus of research since, 1982 when Tsuruo *et al.* (1982) showed that verapamil augments the antiproliferative effect of vincristine. Empirical approaches have, however, resulted in very limited success in the clinic (Borst, 1999; Tan *et al.*, 2000). Our work has therefore focussed on understanding the mechanistic details of the catalytic cycle of ATP hydrolysis by Pgp, both because of its importance in cancer chemotherapy and as a model for ABC transporters in general.

P-GLYCOPROTEIN

The *MDR1*-encoded protein, Pgp, is a 150–170-kDa plasma membrane protein, a member of the ABC superfamily of transporter proteins, and can extrude a range of hydrophobic anticancer drugs from the cell. Molecular probes developed to identify Pgp in cancer tissues also strongly suggest that in acute leukemias, breast cancer, ovarian cancer, head and neck tumors, and non-Hodgkin lymphoma, overexpression of Pgp plays a significant role in MDR in the clinic (Leith *et al.*, 1999; Ng *et al.*, 1998; Trock *et al.*, 1997; van der Zee *et al.*, 1995; Yuen and Sikic, 1994; Zochbauer *et al.*, 1994). The mammalian MDR gene family consists of two members in humans and three in rodents (Borst and Schinkel, 1997). Of the two human genes *MDR1* and *MDR2*, primarily *MDR1* confers drug resistance. The mouse *mdr1* (*mdr1b*) and *mdr3* (*mdr1a*) show a 80% homology with the *MDR1* human gene product and are also capable of conferring resistance to drugs as are their hamster homologues *pgp1* and *pgp2*. Studies with both cultured cells and transient expression systems provide compelling evidence that the substrates interact directly with Pgp. Table I lists some of the agents that interact with Pgp. These include vinca alkaloids, calcium channel blockers, anthracyclines, antiarrhythmics, epipodophyllotoxins, antihypertensives, antibiotics, immunosuppressants, cytotoxic agents, steroid hormones, and HIV protease inhibitors. A major challenge in elucidating the mechanism of Pgp is to understand how a transporter interacts with so many structurally diverse chemical agents. The only feature common to Pgp drug-substrates appears to be that they are all hydrophobic with a molecular mass of 300–2000 Da (Ford and Hait, 1990; Germann, 1996; Sharom *et al.*, 1999) and some carry a positive charge at pH 7. In recent years, Seelig and co-workers have attempted to understand the biophysical characteristics of Pgp substrates and their theoretical models suggest that it may be a function of the spatial separation of electron-donor groups (Seelig, 1998a,b). Several groups have also been engaged in trying to identify the drug-substrate recognition site(s) on Pgp. Substrate analogues that photoaffinity label the Pgp have proved to be invaluable

Table I. List of Selected Substrates and Modulators of P-Glycoprotein

Substrates	Agents that reverse multidrug resistance (Modulators)
Vinca alkaloids: vinblastine and vincristine	Calcium channel blockers: verapamil, dihydropyridines, and azidopine
Anthracyclines: daunorubicin and doxorubicin	Antiarrhythmics: quinine and quinidine
Epipodophyllotoxins: etoposide and teniposide	Antihypertensives: reserpine and yohimbine
Antibiotics: dactinomycin and actinomycin D	Antibiotics: hydrophobic cephalosporins
Other cytotoxic agents: mitomycin, paclitaxel (Taxol), topotecan, colchicine, emetine, gramicidin D, puromycin, and valinomycin	Immunosuppressants: cyclosporin A and FK506
	Steroid hormones: progesterone and megestrol acetate
	HIV protease inhibitors: sequinavir, indinavir, and retanavir
	Anti-alcoholism drugs: disulfiram (Antabuse)

in this effort. The ^{125}I-labeled iodoazylazidoprazoin (IAAP), an analogue of prazosin (Greenberger, 1993); the ^{125}I-labeled iodomycin, an analogue of daunorubicin (Demmer *et al.*, 1997); the ^{3}H-labeled azidopine (Bruggemann *et al.*, 1989); and 6-*O*-[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethylcarbamyl] forskolin (AIPPF), an analogue of forskolin (Morris *et al.*, 1994), have yielded valuable information on the direct interaction of Pgp with its substrates. While the nature of these interactions is still unknown, experiments with these photoaffinity analogues and mutational analysis (Bruggemann *et al.*, 1989, 1992; Hafkemeyer *et al.*, 1998; Loo and Clarke, 2000) indicate that the interaction is probably in the regions of transmembrane segments 4–6 and 10–12.

Pgp is widely distributed in normal human tissues, such as the blood-brain barrier, liver, kidney, intestine, adrenal glands, and testes. This has long prompted speculation on the role of Pgp in normal cells (Ambudkar *et al.*, 1999; Gottesman and Pastan, 1993). On the basis of this tissue distribution several normal physiological roles for Pgp have been speculated in absorption, distribution, and excretion of xenobiotics. There was also a more urgent need to understand the normal physiological role of Pgp. Effective inhibitors of Pgp-mediated drug transport were being developed and entering clinical trials. The safe use of these inhibitors requires that the functions of Pgp in normal physiology are understood to anticipate and eventually limit the potential side effects arising out of their use. Knockout mice with disrupted Pgp genes have provided us with a direct method of studying these functions.

Mice with disruptions of each of the three Pgp-encoding genes (*mdr1a*, *mdr1b*, and *mdr2*) have been obtained (Borst and Schinkel, 1996). The knockout mice, under laboratory conditions, are all healthy, with a normal life span, are fertile, and are normal anatomically and histologically. The most striking result obtained with the mice homozygous for a disrupted *mdr1a* gene was the role of Pgp as an active extruder of molecules that pass the blood-brain barrier. For example, ivermectin, an excellent Pgp substrate, accumulates to levels over 100-fold higher in the brains of *mdr1a*$^{-/-}$ mice than in *mdr1a*$^{+/+}$ mice (Schinkel *et al.*, 1994). The tolerance of *mdr1a*$^{-/-}$ mice to ivermectin was also reduced 100-fold. The results from the double knockout mice (*mdr1a* + *mdr1b*) are particularly important because unlike humans mice have two functional drug-transporting genes and only a double knockout can tell us about the untoward effects that may be expected by the use of powerful MDR reversal agents (Schinkel *et al.*, 1997). The double knockouts show no gross physiological, anatomical, or pathological abnormality. Particularly important is the fact that gross disturbances in corticosteroid metabolism in pregnancy and in bile formation, which could be reasonably expected to occur based on earlier speculations of the function of Pgp, are absent in these mice. These studies suggest a protective role against chemical toxicity for Pgp in mice and possibly in humans. Recent studies with mice deficient in *mdr1a/mdr1b* and *mrp1* and cell lines derived from these triple knockout (*mdr1a/mdr1b*$^{-/-}$, *mrp1*$^{-/-}$) mice suggest that Pgp and MRP1 transporters contribute significantly to the development of resistance to anthracyclines, paclitaxel (Taxol), and vinca alkaloids (Allen *et al.*, 2000). In addition, it appears that both Pgp and MRP1 are compensatory transporters for vinca alkaloids since the exposure of these triple knockout mice to therapeutic doses of vincristine resulted in severe damage to bone marrow and gastrointestinal mucosa (Johnson *et al.*, 2001).

MODELS OF Pgp-MEDIATED DRUG TRANSPORT

The most widely accepted model is that the Pgp, a molecular pump, uses the energy of ATP hydrolysis to extrude chemotherapeutic agents from the cell. According to this hypothesis, chemotherapeutic agents diffuse down a concentration gradient into the cell. The drugs could then be expelled from the bilayer itself or the drugs could be first transported from the cytosol to the membrane and subsequently pumped out from the membrane. The flippase model suggested by Gottesman and Higgins (Higgins and Gottesman, 1992) on the other hand proposes that the drugs are transported from the inner leaflet to the outer leaflet of the bilayer and then extruded. Although an alternative mechanism has been suggested in which changes in the intracellular pH and membrane potential alter the transmembrane partitioning or intracellular sequestering of the drugs (Roepe, 1995), a large body of evidence favors the ATP-dependent active transport model. Drug-binding and photoaffinity studies show direct interaction between Pgp and many of the substrates (Bruggemann *et al.*, 1989; Demmer *et al.*, 1997; Greenberger, 1993; Morris *et al.*, 1994). The drugs stimulate ATPase activity in proportion to the ability of Pgp to transport those drugs (for reviews see Senior, 1998; Senior *et al.*, 1998). Specific amino acid substitutions alter the substrate specificity of Pgp (for reviews see Ambudkar *et al.*, 1999; Gottesman *et al.*, 1995). Additionally, purified Pgp reconstituted into phospholipid vesicles is capable of drug transport even in the absence of electrochemical gradients (Ambudkar, 1998; Ruetz and Gros, 1994; Sharom *et al.*, 1993).

The notion that the energy of ATP hydrolysis is utilized by Pgp to actively pump drugs is central to the active

pump model. Consequently, the substrate-stimulated ATPase activity of Pgp has been studied in considerable detail (Ambudkar *et al.*, 1992; Kerr *et al.*, 2001; Sarkadi *et al.*, 1992; Senior, 1998; Senior *et al.*, 1995; Senior and Gadsby, 1997; Sharom *et al.*, 1993, 1995; Urbatsch *et al.*, 1994). Pgp consists of two transmembrane segments and two ATP-binding sites. Each ATP site is composed of three conserved regions, Walker A motif, Walker B motif, and a hydrophobic dodecapeptide—a signature of ABC superfamily members, also called the linker or C region. ATP hydrolysis is abolished by the chemical modification with *N*-ethylmaleimide (al-Shawi *et al.*, 1994) or mutations in the conserved residues of the Walker A, Walker B, or C region (Hrycyna *et al.*, 1999; Loo and Clarke, 1995a,b;). Orthovanadate (Vi), which behaves as an analogue of inorganic phosphate, inhibits the ATPase by trapping Mg-ADP at the catalytic site. Mutational analysis and chemical modification have also established that both the sites can bind and hydrolyze ATP (Urbatsch *et al.*, 1995a,b). However, the interaction of both sites is essential for ATP hydrolysis and drug transport (Hrycyna *et al.*, 1998, 1999). It has been shown using purified protein that Pgp catalyzes substrate-stimulated ATP hydrolysis (Ambudkar *et al.*, 1992; Shapiro and Ling, 1995; Sharom *et al.*, 1993). The protein, however, also shows basal ATPase activity in the absence of substrate, which may be due to activation by endogenous lipids or peptides (Ramachandra *et al.*, 1996).

INTERACTIONS BETWEEN THE DRUG (SUBSTRATE) AND ATP-BINDING DOMAINS OF Pgp

Crude membrane fractions as well as purified Pgp reconstituted into lipid vesicles manifest a basal level of ATP hydrolysis which is stimulated by substrates and modulators of Pgp by a factor of up to 10 (Ambudkar *et al.*, 1992; Loo and Clarke, 1995a,b; Sarkadi *et al.*, 1992). There is now considerable evidence of drug-stimulated ATPase activity in Pgp from diverse systems suggesting that ATP hydrolysis and drug transport is intimately linked. Several groups have demonstrated that Pgp exhibits a Mg^{2+}-dependent ATPase activity with a single K_m in the 0.3–1 mM range, depending on the source of Pgp (Ambudkar *et al.*, 1992; Kerr *et al.*, 2001; Sharom *et al.*, 1995; Urbatsch *et al.*, 1994). The low affinity of nucleotides for Pgp presents technical difficulties (Urbatsch *et al.*, 1995a,b) which have been overcome in large measure by using Vi to generate a stable noncovalent ternary complex in the form of the transition-state intermediate Pgp·ADP·Vi. Vi is an efficient inhibitor of Pgp ATPase activity because it is similar in size and charge to P_i, readily increases the coordination sphere to five, and exhibits plasticity in its bond distances (Smith and Rayment, 1996). The Pgp·ADP·Vi species is thus expected to mimic the catalytic transition state with P_i during ATP hydrolysis by Pgp (Senior and Gadsby, 1997). It has been established that it is always a nucleoside diphosphate that is trapped (Sankaran *et al.*, 1997; Urbatsch *et al.*, 1995a,b). Thus if ATP (or 8-azidoATP) is used to initiate the reaction, at least one turnover of ATP hydrolysis, converting ATP to ADP, is essential for trapping to occur. Moreover, we have recently demonstrated (Sauna *et al.*, 2001) that it is possible to initiate Vi-induced trapping with either [α-^{32}P]8-azidoADP or [α-^{32}P]8-azidoATP. Trapping by either route showed similar kinetics, similar distribution between the N- and the C-terminal halves of Pgp, and the same requirement for divalent cations and the Vi-trapped intermediate generated with 8-azidoADP, 8-azidoATP, ADP, and ATP resulted in similar drastically reduced binding of the drug-substrate analogue, IAAP to Pgp. The only difference was that the activation energy for generating the Pgp·[α-^{32}P]8-azidoADP·Vi transition-state complex starting with [α-^{32}P]8-azidoADP was approximately 2.5 times greater than if [α-^{32}P]8-azidoADP were used (Sauna *et al.*, 2001).

Using techniques such as Vi-induced trapping and chemical modifications at the ATP sites, Senior and colleagues have dissected the steps involved in ATP hydrolysis by Pgp to propose a catalytic scheme (Senior, 1998; Senior *et al.*, 1995a,b; Senior and Gadsby, 1997). The essential feature of this model is alternating hydrolysis of ATP at the two ATP-binding sites. It is postulated that nucleotide first binds to one of the two sites but cannot be hydrolyzed. When another nucleotide binds to the second site it promotes hydrolysis at the first site, which in turn powers substrate transport. In the next cycle, hydrolysis occurs at the second ATP site. This model is based on the fact that Vi-trapping of the nucleotide at either catalytic site arrests ATP hydrolysis at both sites and that mutations or chemical modifications that inactivate one catalytic site also prevent catalysis at the other site (Hrycyna *et al.*, 1998; Loo and Clarke, 1995a,b; Urbatsch *et al.*, 1998).

Direct and simultaneous kinetic measurements at the drug-substrate and the ATP-sites of Pgp have provided valuable mechanistic details vis-à-vis the catalytic cycle. Pgp exhibits low affinity for ATP compared to, for example, myosin or the mitochondrial F_1F_0-ATP synthase (Sauna *et al.*, 2001; Senior, 1998). Additionally, in Pgp no covalent phosphorylated (E∼P) intermediate has been demonstrated as is known to occur for the P-type ATPases (Lelong *et al.*, 1994; Senior, 1998). These facts led

Senior and co-workers to hypothesize that during ATP hydrolysis a state of high chemical potential is generated and that the relaxation of such a state powers the extrusion of drug-substrate (Senior *et al.*, 1995a,b; Senior and Gadsby, 1997). Recent work from our laboratory experimentally demonstrated a large conformational change accompanying ATP hydrolysis. We showed that the long-lived Pgp·8-azidoADP·Vi transition-state complex, which is generated immediately following ATP hydrolysis, exhibits a drastic decrease in the affinity for the substrate analogue [^{125}I]IAAP (Sauna and Ambudkar, 2000) and also for the nucleotide [α-^{32}P]8-azidoATP (Sauna and Ambudkar, 2001). Thus, conformational changes that follow ATP hydrolysis reduce the affinity for both substrate and nucleotide for Pgp, and the extent of decrease in the affinity for the nucleotide [α-^{32}P]8-azidoADP is comparable to that for the drug-substrate [^{125}I]IAAP. There is nonetheless an important distinction in the events occurring at the drug-substrate and ATP-binding domains. The release of [α-^{32}P]8-azidoADP from the complex is spontaneous and not affected by the presence of excess nucleotides and this is sufficient for the next ATP hydrolysis event to ensue (Sauna and Ambudkar, 2001). However, the release of the occluded 8-azidoADP was not sufficient to regain substrate binding, which occurred only after an additional round of ATP hydrolysis and ADP release (Sauna and Ambudkar, 2000, 2001). Hence, two hydrolysis events occur in a single catalytic cycle, one associated with efflux of drug and the other to bring about conformational changes that "reset" the molecule (Sauna and Ambudkar, 2000, 2001).

THE ALTERNATING HYDROLYSIS OF ATP AT THE TWO ATP-SITES: HOW DOES IT OCCUR?

We have seen above that two hydrolysis events occur during a single catalytic cycle of Pgp. We exploited the fact that it is experimentally possible to propel Pgp through repeating cycles of Vi-induced [α-^{32}P]8-azidoADP trapping and [α-^{32}P]8-azidoADP release to kinetically characterize these two events. The data demonstrated that although the $t_{1/2}$ for each trapping and release events are comparable, the conformational state of the molecule differs during each of these events (Sauna and Ambudkar, 2001). Concurrent measurements of substrate binding and Vi-induced trapping of [α-^{32}P]8-azidoADP distinguish different states of the Pgp molecule in which the two ATP hydrolysis events occur. Thus, when the first hydrolysis event is initiated [^{125}I]IAAP binding is not affected in contrast to over 90% inhibition of [^{125}I]IAAP binding when the second hydrolysis event is initiated. At the end of the second hydrolysis event, however, [^{125}I]IAAP binding is restored to normal levels, bringing Pgp to its initial state both in terms of substrate and nucleotide binding, suggesting the completion of one catalytic cycle. The critical piece of evidence, however, was that the K_m ([α-^{32}P]8-azidoATP) for trapping is identical for the two hydrolysis events. Thus, although these events have different functional consequences, they are kinetically indistinguishable. This would suggest that it is unlikely that the two hydrolysis events are individually associated with each ATP site. We propose that the nucleotide-binding site for any hydrolysis event at high nucleotide concentration is recruited randomly, that all hydrolysis events are kinetically equivalent, and that the different functional outcomes are a result of the conformational state of the Pgp molecule when a particular hydrolysis event occurs.

Earlier studies with plasma membranes from Chinese hamster ovary cells over expressing Pgp clearly showed that the trapped [α-^{32}P]8-azidoADP labels the two ATP sites in equal proportion (Sankaran *et al.*, 1997; Urbatsch *et al.*, 1995a,b). Our results with recombinant human Pgp similarly demonstrate that this distribution remains constant during both the hydrolysis events (Sauna and Ambudkar, 2001). These results strongly favor the conclusion that the two ATP sites are recruited randomly and show similar kinetics. Moreover, the observation that trapping of 1 mol ADP/mol Pgp is sufficient to block ATP hydrolysis (Urbatsch *et al.*, 1995a,b, 2000) has been interpreted to mean that trapping of nucleotide at one site blocks catalysis at both sites (Senior, 1998; Senior *et al.*, 1995a,b; Senior and Gadsby, 1997). This observation has in turn led to the speculation (Senior, 1998; Senior *et al.*, 1995a,b; Senior and Gadsby, 1997) that the binding of nucleotide at one ATP site is not sufficient for hydrolysis to occur and that the binding of nucleotide at the second ATP site permits hydrolysis at the first site by an allosteric mechanism. On the other hand, data presented by us provides direct experimental evidence that following ATP hydrolysis, the affinity of nucleotide for Pgp is drastically reduced (Sauna and Ambudkar, 2001). Thus, the fact that trapping nucleotide at either ATP site blocks hydrolysis at both, can be explained by this conformational change that drastically reduces the affinity of nucleotide for the second ATP site. Such a perspective would also be consistent with the characterization of ATP hydrolysis in Pgp, which shows Henri–Michaelis–Menten kinetics with a single K_m for ATP (Ambudkar *et al.*, 1992; Senior *et al.*, 1995a,b; Sharom *et al.*, 1995), since no cooperativity has been demonstrated

vis-à-vis the kinetics of ATP hydrolysis to suggest allosteric modulation.

THE RATE-LIMITING STEP(S) IN THE CATALYTIC CYCLE OF ATP HYDROLYSIS BY Pgp AND THE EFFECT OF SUBSTRATES OR MODULATORS

There is considerable evidence in the literature that the ATPase activity of Pgp is increased or decreased in the presence of amphipathic drugs, which are substrates or modulators of Pgp (Ambudkar *et al.*, 1992; Ramachandra *et al.*, 1998; Scarborough, 1995; Urbatsch *et al.*, 1994). Although these compounds affect the ATP hydrolysis by Pgp, they have no effect on the values of K_m for nucleotides such as ATP and 8-azidoATP, and the value of K_i for ADP, a competitive inhibitor of ATP hydrolysis, remains constant in the presence of verapamil (Kerr *et al.*, 2001). Additionally, it has been demonstrated that these compounds have no effect on the binding per se of nucleotide to Pgp (Sauna *et al.*, 2001).

We used the Vi-induced trapping of Pgp in the Pgp·[α-^{32}P]8-azidoADP·Vi transition state (see above) to determine the steps of the catalytic cycle of ATP hydrolysis where these drugs exert their effect (Kerr *et al.*, 2001). As Vi mimics P_i, the extent of [α-^{32}P]8-azidoADP trapped into Pgp is comparable to the transition-state conformation Pgp·ADP·P_i of the ATP hydrolysis reaction. Although Vi inhibits ATPase activity of Pgp in steady-state experiments, a very strong linear correlation exists between the extent of [α-^{32}P]8-azidoADP trapped into Pgp in the presence of Vi and the steady-state fold-stimulation of ATPase activity in the presence of various substrates (see Fig. 2).

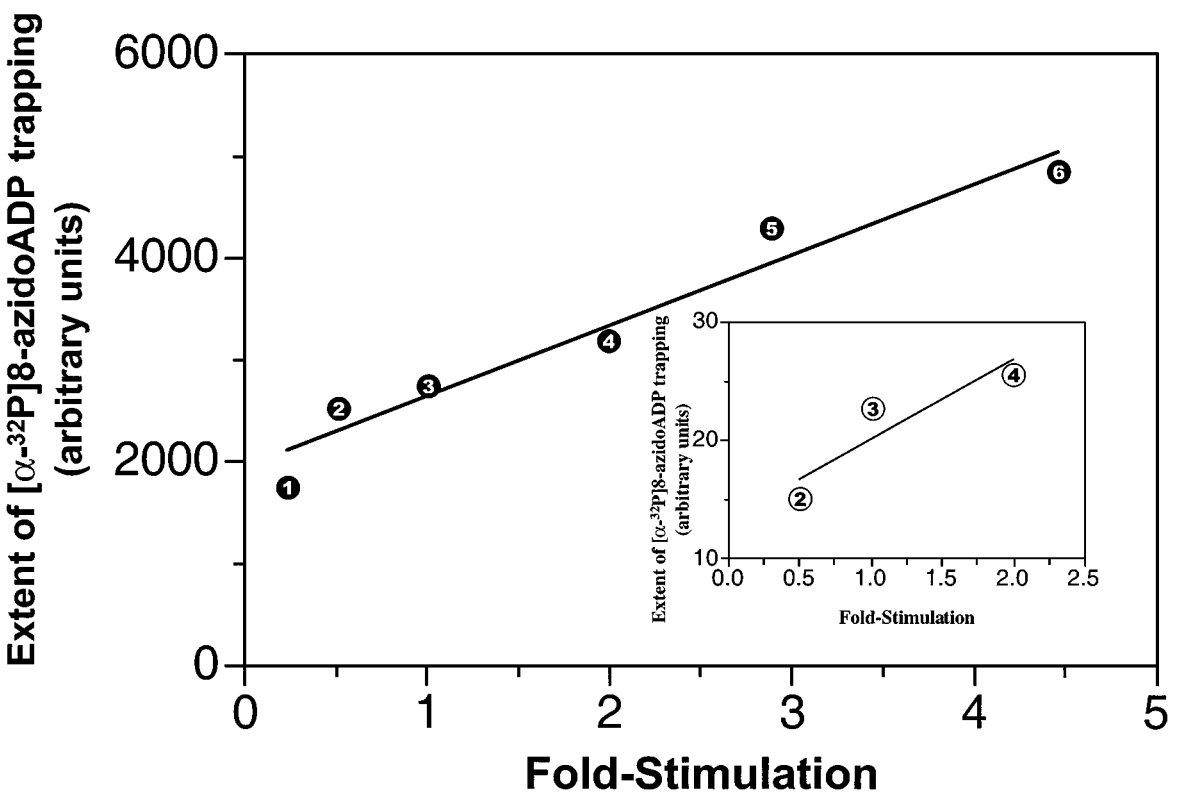

Fig. 2. Correlation between the extent of Vi-induced 8-azidoADP trapping and the fold-stimulation of ATPase activity in the presence of Pgp substrates. The extent of Vi-induced trapping of [α-^{32}P]-8azidoADP after a 15-min incubation at 37°C in the presence of 50 μM [α-^{32}P]-8azidoATP and 5 mM $CoCl_2$ were correlated ($r = 0.97$) to the fold-stimulation of steady-state ATPase activity under saturating MgATP conditions. The numbers represent the data obtained in presence of indicated compound: ❶, rapamycin (20 μM); ❷, cylosporin A (1 μM); ❸, DMSO; ❹, verapamil (50 μM); ❺, valinomycin (5 μM); and ❻, prazosin (100 μM). Inset shows a similar correlation ($r = 0.90$) between Vi-induced [α-^{32}P]-8azidoADP trapping and the fold-stimulation of ATPase activity observed when $CoCl_2$ was replaced with 5 mM $MgCl_2$. The numbers indicate the same compounds as in the main figure, viz., ②, cylosporin A (1 μM); ③, DMSO; and ④ verapamil (50 μM).

Thus, compounds that support a higher fold-stimulation of steady-state ATPase activity also demonstrate a higher extent of Vi-induced [α-^{32}P]8-azidoADP occlusion in the presence of either Co^{2+} or Mg^{2+} into Pgp (Kerr *et al.*, 2001; Szabo *et al.*, 1998). This correlation suggests that both the experimental approaches are measuring the same step in the catalytic cycle of Pgp and that the rate-limiting step in the catalytic cycle is the release of either ADP or P_i.

P_i has an extremely low affinity for Pgp with a K_i in the range of 150–200 mM for ATP hydrolysis. This makes it unlikely that the release of P_i would be the rate-limiting step. Also, the correlation between the steady-state reaction (fold-stimulation) and the amount of [α-^{32}P]8-azidoADP trapped in the presence of substrates indicates that the rate-limiting step is a step after Vi binds and traps Pgp, and P_i release is a prerequisite for Vi binding. Other observations also support the view that ADP release is the rate-limiting step in the catalytic cycle. There is an inverse relationship between ADP release from the Pgp·MgADP·Vi complex and the recovery of the substrate binding to the transporter following the transition-state step (Sauna and Ambudkar, 2000). In addition, the rate of the release of 8-azidoADP (or ADP) from the Vi-trapped Pgp is not affected by the addition of excess nucleotides such as ATP, ADP, or the nonhydrolyzable analogue of ATP, AMPPNP (Sauna and Ambudkar, 2001). Taken together these results indicate that the release of ADP from the Pgp·MgADP·P_i transition state appears to be the rate-limiting step in the catalytic cycle of Pgp. How drugs affect the rate of release of ADP is not well understood at present. It is plausible that the signal transduction from the substrate-binding site to the ATP site via the ABC signature or linker (or C) region of the ATP site in the molecule may be modulated by the substrates.

A PROPOSED MODEL FOR THE CATALYTIC CYCLE OF ATP HYDROLYSIS BY Pgp

Building upon the model proposed by Senior's group (Senior, 1998; Senior *et al.*, 1995a,b; Senior and Gadsby, 1997) we have recently elucidated the catalytic cycle of Pgp in considerable detail (Kerr *et al.*, 2001; Sauna *et al.*, 2001; Sauna and Ambudkar, 2000, 2001). The essential features of the cycle are illustrated in Fig. 3. The drug and ATP first bind to Pgp (Step I), there being no energetic requirement for the drug to bind. The studies of Liu and Sharom (1996) also demonstrate, using the fluorescent probe 2-(4-maleimidoanilino)napthelene-6-sulfonic acid, that prior binding of ATP is not essential for drug interaction with Pgp. Thus, ATP binding could precede, follow, or accompany the binding of drug. The hydrolysis of ATP (or 8-azidoATP) and the attendant release of P_i that generates the Pgp·ADP·Vi complex is accompanied by a large conformational change that (possibly besides other effects) drastically reduces the affinity of substrate for Pgp (Dey *et al.*, 1997; Ramachandra *et al.*, 1998; Sauna and Ambudkar, 2000, 2001). Additionally, drugs do not affect nucleotide binding nor do nucleotides influence the binding of substrate. The binding of nucleotide and drug is followed by the first hydrolysis event (Step II), which is accompanied by a conformational change that reduces the affinity of both substrate (Sauna and Ambudkar, 2000, 2001) and nucleotide (Sauna and Ambudkar, 2001) for Pgp. This intermediate can be trapped by using Vi, an analogue of P_i that generates the stable Pgp·ADP·Vi complex (Step IIIA). Following hydrolyis, ADP is released (Step IV). This release occurs spontaneously and is not influenced by the presence of nucleotides. The dissociation of ADP is accompanied by a conformational change that allows nucleotide binding but substrate binding continues to be reduced. A second ATP hydrolysis event is then initiated (Step V) which is kinetically indistinguishable from the first; at which point the substrate binding is still not regained. This event too can be captured as an intermediate by using Vi to trap the nucleotide (Steps VIA and VIB). The subsequent release of ADP (Step VII) completes one catalytic cycle, bringing the Pgp molecule back to the original state where it can bind both substrate and nucleotide to initiate the next cycle. The conformation of Pgp following ATP hydrolysis shows reduced affinity for the nucleotide (Steps II, III, IIIA, V, VI, and VIA). Additionally, following the second ATP hydrolysis event, the release of ADP from Pgp is essential to complete the catalytic cycle, i.e. to bring the molecule back to the state where it will bind the next molecule of drug-substrate (Steps VI B and VII). Finally, this model is consistent with our recent finding that ADP release at Steps IV and VII (Kerr *et al.*, 2001) appear to be the rate-limiting steps in the catalytic cycle.

This model suggests two unique and distinct roles for ATP hydrolysis in a single turnover of the catalytic cycle of Pgp. Not only is energy utilized in the transport of substrate but there is a clear need for ATP hydrolysis in effecting conformational changes in the molecule that make it available for the next catalytic cycle. This is consistent with the fact that the hydrolysis of at least two molecules of ATP is required for the transport of every molecule of substrate (Ambudkar *et al.*, 1997; Shapiro and Ling, 1998). These values are also similar to that obtained for other ABC transporters (Liu *et al.*, 1997; Mimmack *et al.*, 1989). We have also determined that the two hydrolysis events in a single catalytic cycle are kinetically identical and differ

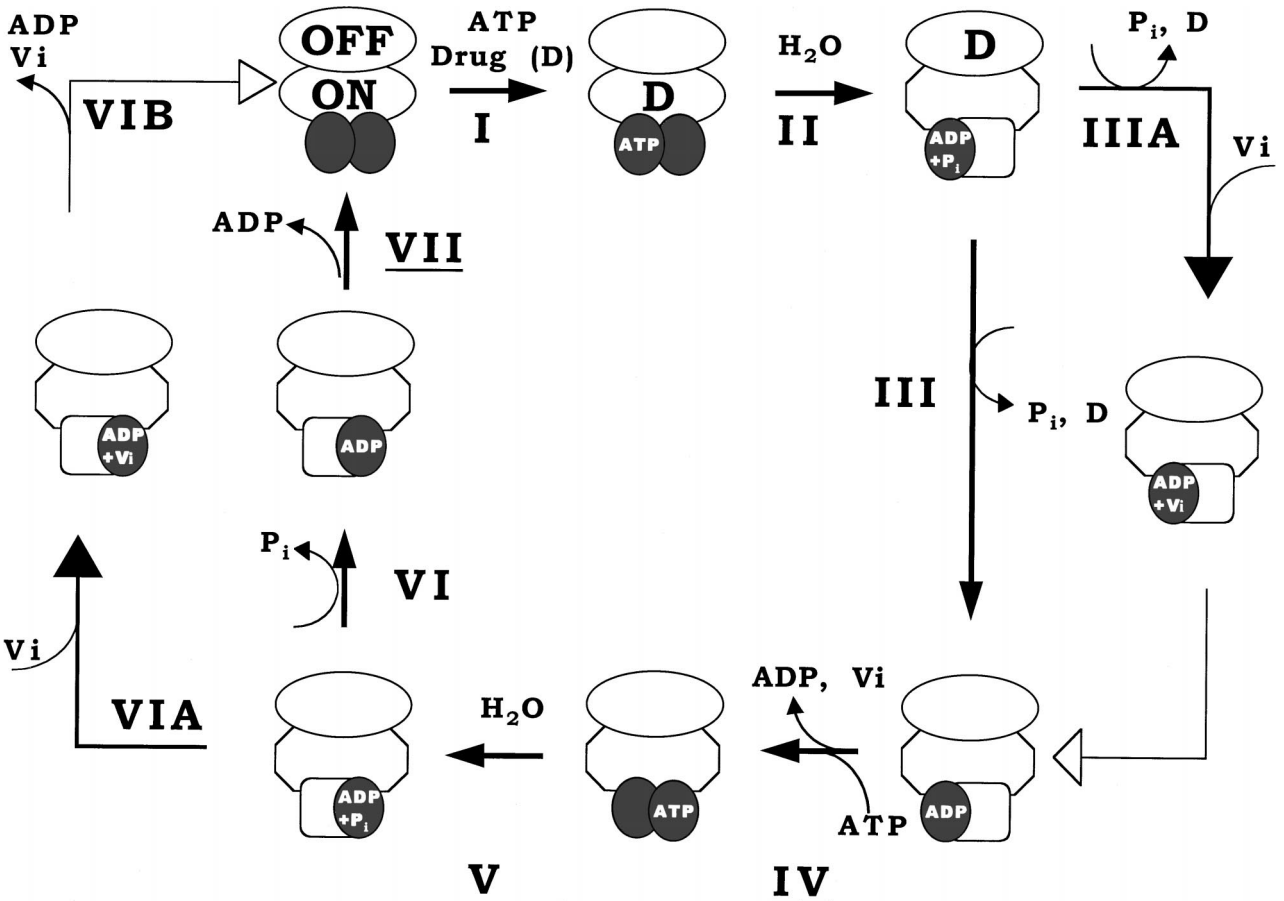

Fig. 3. A proposed scheme for the catalytic cycle of ATP hydrolysis by Pgp. The scheme depicted here is based on published work by us and others (Dey *et al.*, 1997; Kerr *et al.*, 2001; Sauna and Ambudkar, 2000, 2001; Senior, 1998; Senior and Gadsby, 1997; Senior *et al.*, 1995a,b). The ellipses represent the substrate binding sites, the "ON" and the "OFF" sites. The hexagon portrays the "ON" site with reduced affinity for the drug. Two circles represent the ATP sites and the circles are shown overlapping to indicate that both sites are required for ATP hydrolysis. The empty square with rounded edges represents the ATP site with reduced affinity for nucleotide. The release of ADP in Steps IV and VII (underlined) appears to be rate limiting, which is modulated by drug-substrate (Kerr *et al.*, 2001). See text for additional details.

only with respect to the status of the molecule vis-à-vis substrate binding. This too is consistent with the notion that ATP does not show preferential affinity to either ATP site and the sites are recruited randomly for hydrolysis (see above). The recent resolution of the crystal structure of the soluble ATP subunit, ArsA of the bacterial arsenite efflux pump (Zhou *et al.*, 2000), and ATP subunits of bacterial ABC transporters Mut S (Lamers *et al.*, 2000; Obmolova *et al.*, 2000) and Mal K (Diederichs *et al.*, 2000) that exhibit structural and functional similarity to Pgp, show that the two functional ATP sites are each composed of residues from both the N- and the C-terminal ATP sites. Such a tertiary structural organization is plausible for Pgp, where the nucleotide-binding domains in the N- and C-terminal halves of the protein each contribute to both ATP binding and ATP hydrolysis with similar kinetic properties.

CONCLUSIONS

The effective chemotherapy of cancer continues to be hindered by the resistance of tumor cells to chemotherapeutic agents. In the last few years our understanding of the mechanism of action of Pgp and other MDR-linked ABC transporters has expanded considerably. A clear understanding of the kinetic and energetic basis of Pgp function is a powerful tool in the search for effective modulators. The detailed understanding of the catalytic cycle of Pgp also provides a paradigm for ABC transporters in

general. These studies have also involved the development of novel experimental strategies that can be used in conjugation with specific mutants to enhance our understanding of the biochemical and mechanistic aspects of this complex and interesting transport protein.

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