Involvement of the "Occluded Nucleotide Conformation" of P-Glycoprotein in the Catalytic Pathway†

Gregory Tombline, Alma Muharemagić, Lori Bartholomew White, and Alan E. Senior*

Department of Biochemistry and Biophysics, University of Rochester Medical Center, Box 712 Rochester, New York 14642

Received May 25, 2005; Revised Manuscript Received July 14, 2005

ABSTRACT: We found recently that the combined mutation of both "catalytic carboxylate" residues (E552A/E1197A) in mouse P-glycoprotein (Pgp) arrested the protein in an "occluded nucleotide conformation", possibly a stabilized dimer of nucleotide-binding domains (NBDs), that binds MgATP tightly at stoichiometry of 1 mol/mol Pgp [Tombline, G., Bartholomew, L., Urbatsch, I. L., and Senior, A. E. (2004) *J. Biol. Chem. 279*, 31212−31220]. Here, we further examine this conformation in respect to its potential involvement in the catalytic pathway. The occluded nucleotide conformation is promoted by drugs. Verapamil markedly accelerated the rate of tight binding of MgATP, whereas it did not effect the rate of dissociation. Mutations in "Q-loop" residues that are thought to interfere with communication between drug and catalytic sites prevented the occluded nucleotide conformation, as did covalent reagents *N*-ethylmaleimide and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, which are known to inhibit ATP hydrolysis by reacting in catalytic sites. Mutations of Walker A Ser and Lys residues in combination with E552A/E1197A had the same effect, showing that interaction of these conserved residues with MgATP is required to stabilize the occluded nucleotide conformation. We present an enzymatic scheme that incorporates this conformation. We propose that upon initial loose binding of MgATP at two nucleotide-binding domains (NBDs), together with drug binding, the NBDs dimerize to form the occluded conformation, with one tightly bound MgATP committed to hydrolysis. The pathway progresses such that the tightly bound MgATP enters the transition state and is hydrolyzed. This work suggests that small molecules or peptides that interact at the NBD dimer interface might effectively disable Pgp catalysis.

P-glycoprotein (Pgp)[1] (ABCB1) is a plasma-membrane-located transporter that confers multidrug resistance in mammalian cells and is therefore of considerable clinical interest. Using the energy of ATP hydrolysis, Pgp is able to exclude numerous drugs and other hydrophobic compounds from cells by transporting them outward across the plasma membrane. Consisting of a single polypeptide chain of 1280 amino acid residues in humans, it is a member of the ABC transporter superfamily and shows the typical superfamily arrangement of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) in the order [TMD1-NBD1-TMD2-NBD2] along the chain. Both NBDs have been shown to be capable of ATP binding and hydrolysis, and cooperativity between the two NBDs is known to be mandatory for catalysis (*1*). However, the mechanism by which the energy of ATP hydrolysis is utilized to drive drug extrusion remains to be elucidated, and it is of keen interest because intervention in the catalytic mechanism could well lead to methods of circumventing or disabling Pgp action. Recent reviews of the Pgp structure and function may be found in refs *2*−*5*.

An emerging theme in the enzymology of ABC transporters is the idea of the formation of a tight binding interface between the two NBDs, or "NBD dimerization" as it is often referred to, at one step of the catalytic sequence. NBD dimers have been seen in X-ray structural studies of disparate ABC transporters (*6*−*9*) and have been characterized also in biochemical studies (*10*−*13*). Mutation of the "catalytic glutamate" residue, which in ABC transporters lies immediately C-terminal to the "Walker B" Asp residue, has been used to facilitate formation of the NBD dimer conformation (*7*, *11*). Such mutations (Glu to Gln or Ala) inactivate ATP hydrolysis (*10*−*12*, *14*−*16*). This, together with the observation that the NBD dimer species is seen to contain bound ATP straddling the dimer interface (*6*−*8*) suggests that the tight NBD dimer conformation forms after initial binding of ATP at the two NBDs, but precedes the hydrolysis step. At the time of writing this paper, no X-ray structural information on P-glycoprotein had been published. However, a mutagenesis study of Pgp indicated the occurrence of a "closed" conformation involving interdigitation of the two NBDs at the stage of catalytic transition-state formation (*17*) and later cross-linking studies showed the spatial proximity of conserved residues in NBD1 and NBD2 (*18*, *19*). Recent electron microscopy studies also show that the NBDs of Pgp are close to each other (*20*, *21*).

Combined mutation of the catalytic glutamates in NBD1 and NBD2 of human MDR1 Pgp was shown to generate a conformation capable of tight binding of 8-azido-ADP (*15*).

† This work was supported by NIH Grants GM50156 to A.E.S. and T32 CA09363 (Postdoctoral Training Support) to G.T.

* To whom correspondence should be addressed. Telephone: 585-275-6645. Fax: 585-271-2683. E-mail: alan_senior@urmc.rochester.edu.

[1] Abbreviations: Pgp, P-glycoprotein; TMD, transmembrane domain; NBD, nucleotide-binding domain; Vi, vanadate anion; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; DM, *N*-dodecyl-*β*-D-maltoside.

10.1021/bi0509797 CCC: $30.25
Published on Web 08/30/2005

Subsequent studies in our laboratory using mouse MDR3 Pgp[2] in which both catalytic glutamates were mutated to Ala (E552A/E1197A mutant) showed that pure mutant protein, which had very low ATPase activity, bound ATP and ADP tightly in a Mg-dependent, vanadate (Vi)-independent manner, with K_d values of 9.2 and 92 μM, respectively (*22*). This was in marked contrast to wild-type Pgp, which binds MgATP only loosely (K_m MgATP = 0.34 mM, K_d MgATP = 0.4 mM, refs *23* and *24*) and is seen to bind MgADP tightly only if Vi is included to trap a transition-state-like complex (*25*). Binding of MgATP to E552A/E1197A Pgp was very temperature-sensitive (*22*). It was apparent therefore that combined mutation of the two catalytic glutamates in Pgp arrests the enzyme in a conformation, possibly a stabilized NBD dimer, which occludes nucleotide and shows preferential binding of ATP over ADP.

In this paper, we have characterized the occluded nucleotide conformation in mouse MDR3 E552A/E1197A Pgp in respect to its involvement in the catalytic pathway. Earlier work (*15*, *22*) had indicated that formation of the occluded nucleotide conformation was drug-sensitive. Moreover, the maximum stoichiometry of tight nucleotide binding seen under the conditions used (*22*) was 1 mol/mol. Here, we have investigated these two aspects further. We have also investigated the effects of the covalent ATPase inhibitors NBD-Cl and NEM on tight nucleotide binding, and we have used E552A/E1197A Pgp as a template upon which to incorporate further mutations, at conserved residues involved in catalysis, to elucidate the role of these residues at the stage of the occluded nucleotide conformation.

EXPERIMENTAL PROCEDURES

Materials. [8-^{14}C]ADP and [α-^{32}P]ATP were purchased from Perkin−Elmer Life Sciences. 8-Azido-[α-^{32}P]ATP was purchased from Affinity Technologies, Inc. *Escherichia coli* lipids were purchased from Avanti Polar Lipids.

Construction of Mutant Pgp: Expression of Mutant Pgp in Pichia pastoris. Construction of E552A/E1197A Pgp was described in ref *22*. Triple mutants containing E552A/E1197A plus an additional mutation were constructed by one of two approaches. In some cases, the additional mutation could be transferred on a restriction fragment directly from a pre-existing plasmid (see refs *14*, *17*, *23*, and *26*) into the pHILD-mdr3.6-His$_6$ plasmid containing E552A/E1197A mutations (*22*). Where this was not possible, a PCR fragment was generated using a pre-existing plasmid containing the required additional mutation as the template, with forward and reverse PCR primers that incorporated the E552A or E1197A mutation, as described in ref *22*. The additional mutation in combination with either E552A or E1197A could then be transferred into plasmid pHILD-mdr3.6-His$_6$-E552A/E1197A on a restriction fragment (*22*). Expression of mutant Pgp in the yeast *P. pastoris* was performed as described previously (*27*, *28*).

Purification and Quantitation of Pgp. Strains of *P. pastoris* expressing mutant Pgp were grown in fermentor culture and purified as described (*17*, *27*). The Pgp concentration was determined as previously described (*23*). Briefly, increasing amounts of mutant Pgp (0.2−2.0 μg) were subjected to SDS-gel electrophoresis on 10% gels alongside a similar set of samples of a reference pure wild-type preparation, whose concentration had previously been accurately determined by amino acid analysis. Gels were stained with Coomassie Blue and scanned. Protein bands were quantified with Scion Image software (Scion Corporation, Frederick, MD).

Activation of Pgp by Lipids and DTT: Centrifuge Column Elution of Pgp. For activation, Pgp was incubated with DTT (8 mM) and *E. coli* lipid (Avanti, acetone/ether precipitated) at a final ratio of 2:1 lipid/protein (w/w) for 20 min at room temperature followed by sonication for 30 s at 4 °C in a bath sonicator (*27*). Centrifuge column elution of Pgp was exactly as in ref *25*. Briefly, elution was at 4 °C, using 1 mL Sephadex G-50 columns equilibrated in 50 mM Tris-HCl at pH 7.5 and 0.001% DM. As noted in ref *25* and confirmed here, recovery of Pgp was routinely 70−90% as judged by ATPase and/or protein assays. To reduce the amount of experimental manipulation to manageable proportions, we routinely assumed a protein recovery of 75%.

Incubation of Pgp with Nucleotide in Binding Experiments: (a) [α-^{32}P]ATP-Binding Experiments. A total of 5−10 μg of protein that had been activated with DTT and *E. coli* lipids as above was incubated at 37 °C for 20 min with Mg-[α-^{32}P]ATP (concentrations as described in the captions of the figures) in 40 mM Tris-HCl at pH 7.4, 0.1 mM EGTA, and 2.0 mM $MgSO_4$, in a total volume of 50 μL. Next, samples were placed on ice, 50 μL of ice-cold buffer was added, and then the whole 100 μL was immediately passed through a centrifuge column as above.

(b) [8-^{14}C]ADP-Binding Experiments. The same procedure was followed except the incubation was for 120 min at 37 °C. Previous experiments had indicated that the longer time is needed to attain saturation with ADP (*22*).

Effects of Preincubation with NBD-Cl and NEM on Nucleotide Binding. Pgp was first passed through a centrifuge column as above to remove 2-mercaptoethanol in the storage buffer and then activated with lipids (2:1) and 1 mM Tris-(2-carboxyethyl)phosphine HCl (replacing DTT) as above. The Pgp was then incubated at 37 °C for 20 min in the dark with NEM or NBD-Cl (both 250 μM final concentration). Controls had only H_2O (NEM) or DMSO (NBD-Cl). Then, radioactive nucleotide and $MgSO_4$ were added, and nucleotide-binding experiments were carried out as described above.

8-Azido-[α-^{32}P]ATP Photolabeling Experiments. These experiments were carried out as in ref *17*. A total of 2 mM $MgSO_4$ was present throughout. After SDS-gel electophoresis, gels were dried and scanned on a phosphorimager.

RESULTS

Attempts To Increase the Stoichiometry of Tight Nucleotide Binding to E552A/E1197A Pgp. In our earlier study, the maximum stoichiometry of tight MgATP or MgADP binding attained with E552A/E1197A Pgp was 1 mol/mol (*22*). The technique used to measure tight binding in that work consisted of preincubation of Pgp with a fixed concentration of radioactive MgATP (200 μM, 20 min, and 37 °C) or MgADP (200 μM, 120 min, and 37 °C) followed by passage through a centrifuge column and counting of the eluates. Tightly bound nucleotide is defined empirically as the

[2] Mouse MDR3 Pgp is 87% identical to human MDR1 Pgp in sequence.

nucleotide retained after passage through the centrifuge column. As discussed earlier (*22*), under the centrifuge column elution conditions used, the ligand would have to dissociate from Pgp with $k \leq 0.006$ s^{-1} for ≥90% to be retained. Where present, the verapamil concentration was 150 μM, which is optimal for stimulation of ATPase (*28*, *29*). Here, we varied these conditions by increasing the MgATP or MgADP concentrations to 1 mM, the verapamil concentration to 1 mM, and also by extending the time of preincubation. In no case was the stoichiometry of tight nucleotide binding increased above 1 mol/mol (data not shown).

Effects of Verapamil on Rates of Association and Dissociation of Tightly Bound MgATP in E552A/E1197A Pgp. It was found in ref *22* that verapamil at fixed 150 μM concentration increased the stoichiometry of MgATP and MgADP binding when measured after a standard incubation period of 20 min (for MgATP) or 120 min (for MgADP). It seemed likely therefore that verapamil was accelerating the association rate or decreasing the dissociation rate of the nucleotide. Figure 1A shows a typical experiment in which the degree of tight MgATP binding was measured at varying times in the presence or absence of 150 μM verapamil. It is clear that verapamil accelerated the apparent association rate considerably. Values of $t_{1/2}$ for MgATP binding were 1.5 and 28 min, in the presence and absence of verapamil, respectively.[3] Triplicate experiments were performed and gave excellent agreement. Figure 1B shows a typical experiment in which the E552A/E1197A Pgp was first loaded with MgATP by preincubation in the absence of verapamil. After passage through a centrifuge column to remove unbound MgATP, the rate of release of bound nucleotide was followed either in the presence or absence of added verapamil (150 μM) by continuing the incubation then passing samples through centifuge columns at varying times. The curves in Figure 1B show that verapamil had no effect on the rate of release of MgATP. $t_{1/2}$ values were 20.6 and 19.9 min in the presence and absence of verapamil, respectively, and triplicate experiments gave excellent agreement. It is therefore apparent that verapamil has a large effect on the formation of the occluded nucleotide state but no effect on its dissipation.

Effects of a Variety of Drugs on Stoichiometry of Tight MgATP Binding. Figure 2 shows the stoichiometry of tight MgATP binding after preincubation of E552A/E1197A Pgp in the presence of various drugs. The maximum stoichiometry of MgATP binding seen was 1 mol/mol, with drugs such as verapamil, vinblastine, and valinomycin. Other drugs gave somewhat lower stoichiometry, but it is notable that almost all of these ligands increased the binding of MgATP over that measured in the control without any drug added.

Covalent Reaction of E552A/E1197A Pgp with NBD-Cl or NEM Impairs Formation of the Occluded Nucleotide Conformation. NBD-Cl and NEM were previously shown to be effective covalent inhibitors of Pgp ATPase activity (*29*, *30*). It was therefore of interest to study their effects on tight binding of MgATP in the occluded nucleotide conformation. Figure 3 shows effects of preincubation of E552A/E1197A Pgp with the two reagents on tight MgATP binding. Both reduced tight MgATP-binding capability considerably. Because labeling by the reagents was probably not fully stoichiometric under the conditions used (to avoid nonspecific labeling), it could be that stoichiometrically labeled protein would show even less tight binding of MgATP.

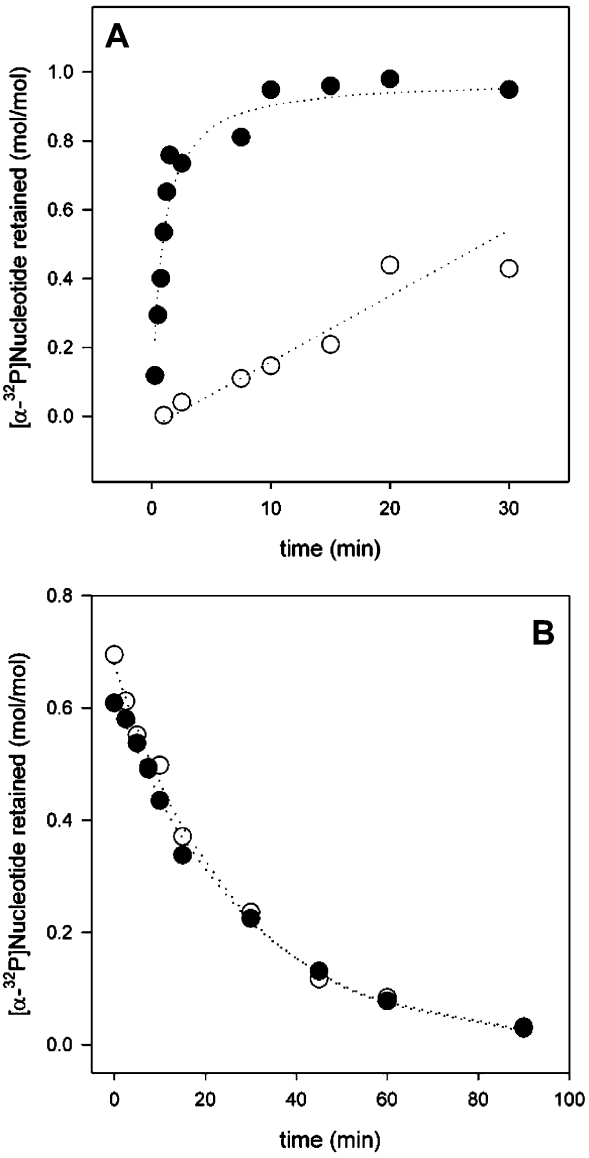

FIGURE 1: Rates of association and dissociation of MgATP in E552A/E1197A Pgp in the presence and absence of verapamil. (A) Mg-[α-^{32}P]ATP (200 μM) was incubated with Pgp for varied times as indicated, in the presence (●) or absence (○) of 150 μM verapamil at 37 °C. Samples were passed through centrifuge columns to separate bound from free ligand, and bound Mg-[α-^{32}P]ATP was estimated by Cerenkov counting. For further details, see the Experimental Procedures. (B) Pgp was incubated with Mg-[α-^{32}P]ATP (200 μM) for 90 min at 37 °C in the absence of verapamil. The sample was passed through a centrifuge column to separate bound from free ligand. At this point, the amount of Mg-[α-^{32}P]ATP bound was 0.62−0.72 mol/mol Pgp. Eluates were incubated for further time at 37 °C in the presence (●) or absence (○) of 150 μM verapamil and then passed through a second centrifuge column to estimate the bound nucleotide remaining.

Using E552A/E1197A Pgp as a Template upon which to Add Mutations at Important, Conserved Residues. In this section of the work, we combined additional mutations with the two existing mutations in E552A/E1197A Pgp and then

[3] The data reported here agreed exactly with the same experiment (in the presence of verapamil) reported in Figure 4B of ref *22*; however, because of a typographical error, the $t_{1/2}$ was reported as 3.0 min in ref *22*.

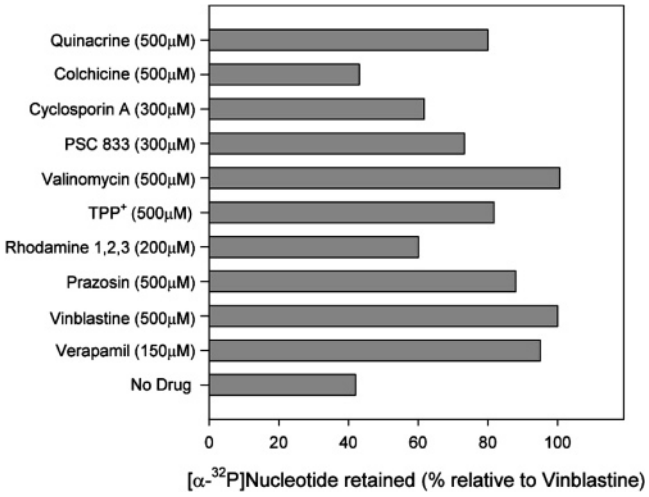

FIGURE 2: Effects of various drugs and drug-site ligands on tight MgATP binding by E552A/E1197A Pgp. Pgp was incubated for 20 min at 37 °C with Mg-[α-^{32}P]ATP (200 μM) in the presence of the drug as indicated and then passed through centrifuge columns to allow an estimation of bound nucleotide. For further details, see the caption of Figure 1 and the Experimental Procedures. In these experiments, the stoichiometry of binding of the nucleotide in the presence of vinblastine was 1.0 mol/mol Pgp and was set at 100%. Results are means of at least triplicate experiments, which showed excellent agreement. Variation from the mean was always ≤10%.

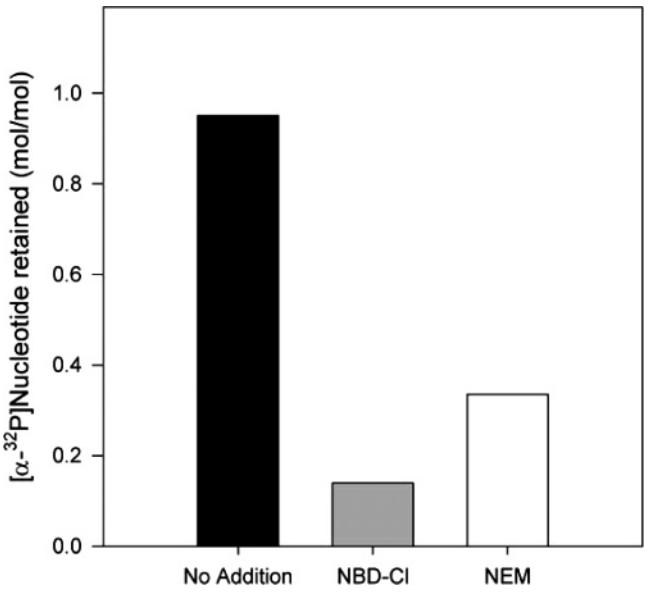

FIGURE 3: Effects of NEM and NBD-Cl on tight binding of MgATP by E552A/E1197A Pgp. Pgp was prereacted with NEM and NBD-Cl (250 μM) for 20 min at 37 °C, then incubated with Mg-[α-^{32}P]-ATP (200 μM) in the presence of 150 μM verapamil for 20 min at 37 °C, and then passed through centrifuge columns to allow an estimation of bound nucleotide. See the Experimental Procedures for details. Controls had DMSO (for NBD-Cl) or H_2O (for NEM). Results are means of at least triplicate experiments, which showed excellent agreement. Variation from the mean was always ≤10%.

assayed effects of the additional mutations on tight MgATP and MgADP binding. All of the triple mutant proteins were expressed in *P. pastoris*, grown in fermenter culture, and could be purified in a similar yield to E552A/E1197A and wild-type Pgp. Each showed the same degree of purity on SDS gels as that of the wild type.

E552A/E1197A/S430A and E552A/E1197A/S1073A. Residues Ser-430 and Ser-1073 in mouse MDR3 Pgp are the "Walker A" serine residues. These residues make direct

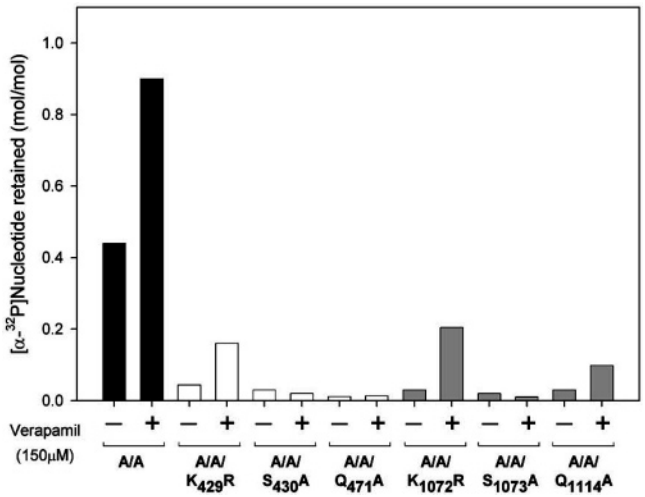

FIGURE 4: Tight binding of MgATP to mutant Pgp. MgATP binding was measured after a 20 min incubation at 37 °C with 200 μM Mg-[α-^{32}P]ATP. When present, the verapamil concentration was 150 μM. At the end of incubation, samples were passed through centrifuge columns to estimate tightly bound nucleotide. "A/A" indicates E552A/E1197A Pgp. Results are means of at least triplicate experiments, which showed excellent agreement. Variation from the mean was always ≤10%. Identical results were obtained when the time of incubation was extended to 60 and 120 min.

contact with the magnesium in MgATP in X-ray structures of numerous nucleotide binding proteins, including ABC transporters (*6*,*31*−*34*). Previous work has shown (*17*) that the single mutations S430A and S1073A strongly impair ATPase activity and abolish Vi-trapping of nucleotide in Pgp, although MgATP binding as evaluated by photolabeling with Mg-8-azido-ATP was unimpaired. Thus it was concluded earlier (*17*) that "the Ser-OH is critical for MgATPase activity and formation of the normal transition state, although not for initial MgATP binding." Figure 4 shows that the mutations S430A or S1073A, when combined with E552A/E1197A, abolished tight binding of MgATP. Figure 5 shows that they had the same effect on tight MgADP binding. Increasing verapamil or nucleotide concentrations to 1 mM, or incubation for longer times, did not change the results. With these mutants, as with all of the mutants described in Figures 4 and 5, presence of Vi had no effect on tight nucleotide binding.

E552A/E1197A/K429R and E552A/E1197A/K1072R. Lys-429 and Lys-1072 in mouse MDR3 Pgp are the Walker A lysine residues that are known to interact with the β or γ phosphates of bound MgATP in X-ray structures of numerous nucleotide-binding proteins, including ABC transporters (*6*−*8*, *31*−*34*). Previous work showed (*14*) that mutation of either Lys-429 or Lys-1072 to Arg in mouse MDR3 Pgp strongly impaired ATPase activity and abolished Vi trapping of the nucleotide. Initial MgATP binding, as evaluated by photolabeling with Mg-8-azido-ATP, was not impaired. Here, it was seen that the combination of K429R or K1072R with E552A/E1197A greatly reduced but did not eliminate completely the tight binding of MgATP or MgADP (Figures 4 and 5). Increasing the verapamil and/or nucleotide concentrations to 1 mM or incubation for longer times did not appreciably increase the stoichiometry of nucleotide binding.

E552A/E1197A/Q471A and E552A/E1197A/Q1114A. Residues Gln-471 and Gln-1114 are found in the "Q-loop" in the NBDs of ABC transporters, postulated from X-ray

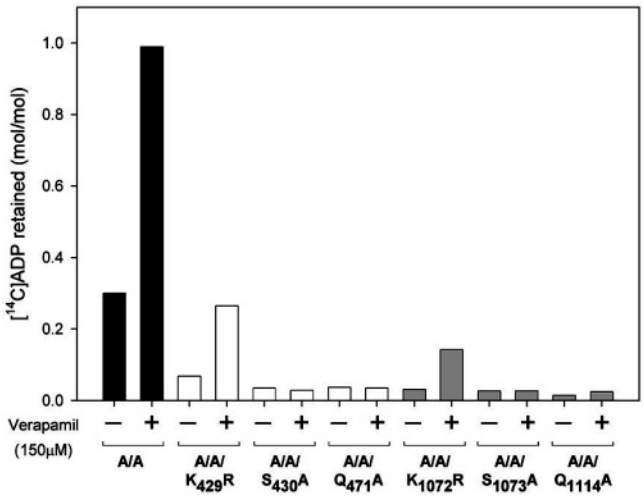

FIGURE 5: Tight binding of MgADP to mutant Pgp. MgADP binding was measured after a 120 min incubation at 37 °C with 200 μM 8-[^{14}C]MgADP. When present, the verapamil concentration was 150 μM and the Vi concentration was 200 μM. At the end of incubation, samples were passed through centrifuge columns to estimate tightly bound nucleotide. "A/A" indicates E552A/E1197A Pgp. Results are means of at least triplicate experiments, which showed excellent agreement. Variation from the mean was always $\leq$10%. Identical results were obtained when the time of incubation was extended to 60 and 120 min.

structural studies to be involved in catalysis either by orienting the catalytic water, by binding the magnesium in the MgATP complex, or by facilitating interdomain communication between transport substrate-binding sites and ATPase catalytic sites (*6*−*8*, *36*). Mutagenesis studies previously indicated that a major role of these residues in Pgp is to facilitate communication between drug sites in the TMDs and catalytic sites in the NBDs (*26*). Here, we observed (Figures 4 and 5) that either Q471A or Q1114A in combination with E552A/E1197A strongly impaired tight binding of MgATP and MgADP. Increasing the time of incubation or concentrations of verapamil and/or nucleotide to 1 mM had no effect.

Photolabeling of the Mutant Pgp Proteins by 8-Azido-ATP. One simple explanation of the inability to bind MgATP or MgADP tightly, as seen in several of the triple mutants in Figures 4 and 5, could be that the proteins were misfolded, although given that the mutant proteins all purified from the membranes of *P. pastoris* in a similar yield and purity to the wild type, this seemed unlikely. We tested this possibility by incubating with Mg-8-azido-[α-^{32}P]ATP at increasing concentrations, photolabeling with UV light, and subjecting the labeled proteins to SDS-gel electrophoresis and phosphorimaging. 8-Azido-ATP has been shown to be a specific probe of ATP binding in Pgp (*14*, *17*, *30*), and it is commonly used in studies of mutant Pgp to assess ATP-binding propensity. It is a good analogue of natural ATP in wild-type Pgp in that it is hydrolyzed well and has a similar K_m to ATP (*30*). It reacts covalently with the conserved Tyr residues in NBD1 and NBD2 of Pgp (*35*) which, in other ABC transporters, were shown by X-ray structural studies to be stacked against the adenine ring of bound nucleotide (*7*, *8*, *36*).

Figure 6 shows the results of this study. It is clear that all of the mutant proteins, even those that did not bind MgATP or MgADP tightly in the experiments of Figures 4 and 5, were able to bind and be covalently labeled by 8-azido-ATP. When allowance was made for varying amounts of protein samples applied in Figure 6 (determined by separately staining the gels with Coomassie Blue), it was evident that there was no difference in labeling intensity between the wild type and any of the mutants and that all of the mutants showed the same response to increasing 8-azido-ATP concentration as in the wild type. In all cases, labeling was strongly competed by inclusion of 10-fold and eliminated by 100-fold excess of MgATP (data not shown). Lack of tight nucleotide binding in Figures 4 and 5 is therefore not due to misfolding of mutant proteins.

DISCUSSION

In an earlier paper (*22*), we demonstrated that mutation of the two catalytic glutamate residues in NBD1 and NBD2 of mouse MDR3 P-glycoprotein to Ala (E552A/E1197A mutant) arrested the enzyme in a conformation, possibly a stabilized NBD dimer, which occludes Mg-nucleotide, shows a preferential tight binding of ATP over ADP, and hydrolyses ATP and releases ADP very slowly. Impairment of turnover is primarily due to the inability to form the normal transition state rather than to slow ADP product release. Here, we study the possible involvement of the occluded nucleotide conformation in normal Pgp catalysis.

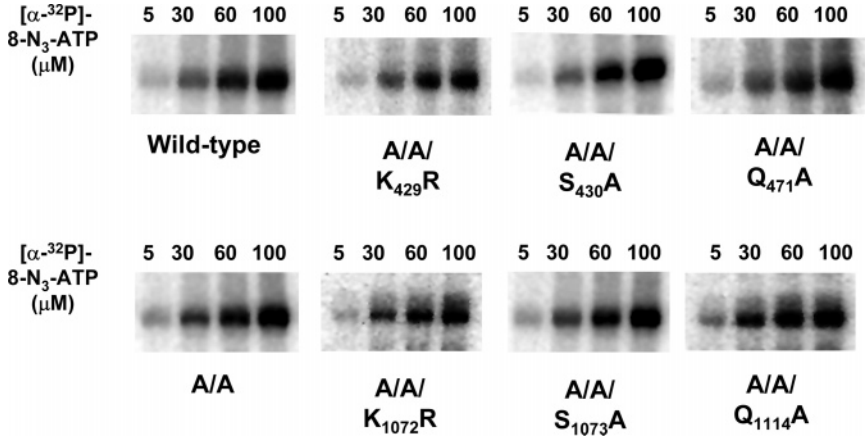

FIGURE 6: Photolabeling of the mutant Pgp by 8-azido-ATP. Samples of Pgp were preincubated with [α-^{32}P]-8-azido-ATP at concentrations shown in the presence of 2 mM $MgSO_4$, subjected to UV irradiation, and then electrophoresed on SDS gels. Photolabeling was detected by phosphorimaging. For further details, see the Experimental Procedures. "A/A" indicates E552A/E1197A Pgp.

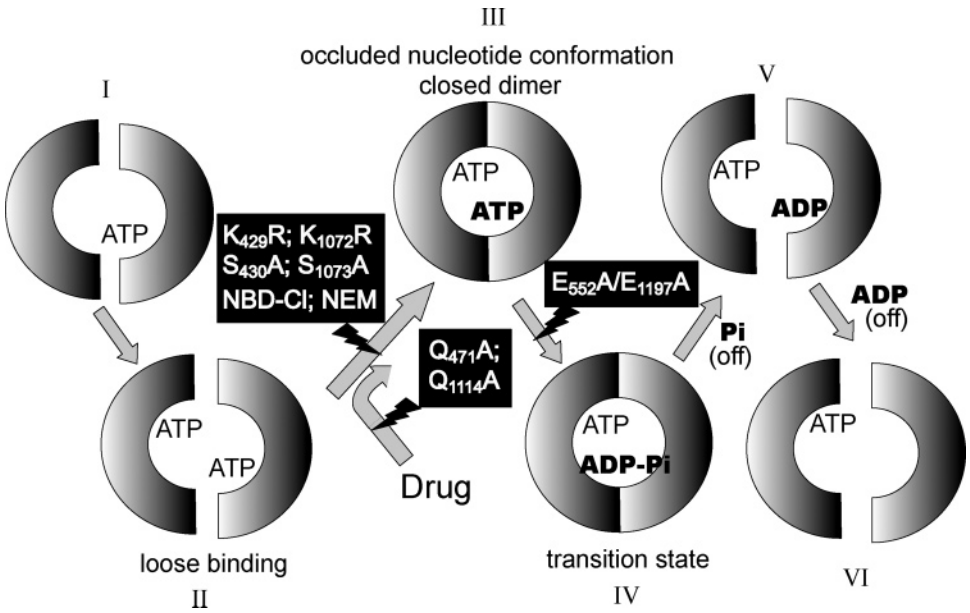

FIGURE 7: Catalytic pathway of Pgp and the involvement of the occluded nucleotide conformation. We propose a model for the Pgp catalysis involving the occluded nucleotide conformation. The catalytic pathway is shown as a series of intermediates I−VI. The diagram shows the occluded nucleotide conformation as an NBD dimer, with the ATP that is bound tightly and committed to hydrolysis in bold. Steps that are suggested to be blocked by mutations, NEM or NBD-Cl, are indicated, as also is the acceleration by drug binding. The diagram is discussed in more detail in the text.

Fluorescence studies have established that initial binding of MgATP occurs loosely ($K_d \sim 0.4$ mM) at two sites in Pgp (*24*, *37*). Other biochemical approaches confirm that both NBDs bind nucleotide (*30*, *38*−*40*). One issue that was apparent in our earlier paper (*22*) was the fact that, using the centrifuge column assay as a criterion for tight nucleotide binding, we found that in the E552A/E1197A mutant the stoichiometry of the tightly bound nucleotide was only 1 mol/mol. This occurred despite the fact that biochemical and X-ray crystallography studies of dimeric ABC transporters (*6*−*8*, *10*−*12*) might more reasonably have predicted a stoichiometry of 2 mol/mol. However, as we discussed (*22*), those structural studies were done on isolated NBD subunits of ABC transporters. Where intact (with TMDs present) transporters were used, as in BmrA (*16*) and our own work (*22*), a stoichiometry of 1 mol/mol was found, leading us to suggest (*22*) that, in the intact transporters, after the initial filling of two sites by ATP, the NBD dimer has a greater tendency to move toward an asymmetric state in which only one of the two nucleotides becomes tightly occluded.[4] It is relevant that in a recent computer simulation of the dynamics of the MgATP-binding process in intact transporter BtuCD (*41*) one nucleotide site becomes progressively more occluded than the other. Here, we experimentally increased concentrations of MgATP, MgADP, and verapamil and increased times of incubation, to try to increase stoichiometry of the occluded nucleotide. However, it was clear that 1 mol/mol was the maximal stoichiometry of tight binding of nucleotide in the E552A/E1197A mutant. Vi-trapping experiments consistently show that in fully inhibited wild-type Pgp, maximally 1 mol of Vi-MgADP (representing the transition state) is trapped per mol of Pgp (*42*, *43*). A recent X-ray structure of MsbA (an ABC transporter homologous to Pgp) with transport substrate bound to the TMDs, shows Vi-MgADP trapped in just one of the two NBDs (*44*). Taking this body of evidence together, we propose that, after the initial loose ($K_d \sim 0.4$ mM) binding of MgATP at both NBDs in Pgp, the occluded nucleotide conformation forms with MgATP tightly bound ($K_d = 9$ μM) at one site, and we hypothesize that this MgATP is committed to enter the transition state and be hydrolyzed.

In parts A and B of Figure 1, we established that the presence of verapamil in the drug-binding site greatly increased the rate of occlusion of MgATP. Therefore, we propose that NBD dimerization is accelerated by occupation of the drug site(s). Verapamil is well-known to stimulate ATPase activity and the rate of Vi-ADP trapping. Both of these effects can be explained by increased rate of formation of the occluded nucleotide conformation and of NBD dimerization. As shown in Figure 2, a range of other Pgp transport substrates also increased occlusion of MgATP in the E552A/E1197A mutant.

NEM and NBD-Cl are covalent inhibitors of Pgp-ATPase activity that react specifically with the Cys residues in the Walker A sequences of Pgp (*39*, *45*) (Cys-427 and Cys-1070 in mouse MDR3 Pgp). Both prevent Vi-ADP trapping, indicating the inability to form the transition state after the reaction (*38*, *46*). Further, it has been shown by Sharom and colleagues (*24*, *37*, *47*) that the fluorescent probe 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS), which reacts with the same Cys residues in the Walker A sequences as NEM and NBD-Cl, also inactivates Pgp ATPase and prevents Vi trapping of nucleotide, yet it has no effect on the loose binding of MgATP at both NBDs that occurs with K_d(MgATP) = 0.4 mM, as measured by fluorescence assay. Also, it was found that reaction with NBD-Cl did not prevent photolabeling by 8-azido-ATP (*38*). Here, we found that reaction with NEM or NBD-Cl impaired tight binding of MgATP in the E552A/E1197A mutant. The data suggest an

[4] It should be noted that cyclotetravanadate, introduced as a heavy atom derivative into crystals of BtuCD (*9*), was seen in both NBDs; however, it is not clear to what extent this species mimics a nucleotide.

explanation of the inhibitory effects of the two reagents; i.e., they prevent formation of the occluded nucleotide conformation by NBD dimerization. The results also implicate the occluded nucleotide conformation as a step in the catalytic pathway.

Using the E552A/E1197A mutant as a template, we incorporated extra mutations to find out how they affected tight nucleotide binding. It may first be stated that in all cases the binding and covalent reaction with 8-azido-[^{32}P]ATP was retained with similar concentration dependence to that seen in the wild type (Figure 6). This showed that the new mutant proteins were not misfolded. The mutants S430A and S1073A at the loci of the Walker A Ser residues in Pgp remove the hydroxyl side chain known to coordinate to the Mg cation of bound MgATP and MgADP in many nucleotide-binding proteins, including ABC transporters (*6*, *31*−*34*). As noted in the Results, the single mutations S430A and S1073A had been shown previously (*17*) to inhibit Pgp ATPase activity and transition-state formation (as measured by Vi-ADP trapping) but not to prevent the initial binding of MgATP (as evaluated by 8-azido-ATP labeling), as if the Ser-OH only became tangibly engaged with the Mg-nucleotide at the stage of a "closed conformation". We had also previously found that tight binding of ATP and ADP in the "occluded nucleotide conformation" of E552A/E1197A Pgp was Mg-dependent (*22*). Consistent with these data, the mutants E552A/E1197A/S430A and E552A/E1197A/S1073A were deficient in tight binding of MgATP and MgADP (Figures 4 and 5). Thus, structural stabilization of the occluded nucleotide conformation (and presumably the NBD dimer) requires interaction between the Walker A Ser side-chain hydroxyl and the Mg cation. Similar arguments may be advanced for the two Walker A Lys residues in Pgp, which likely interact with the β and γ phosphates of the bound nucleotide, as deduced from structural studies of numerous nucleotide binding proteins and ABC transporters (*6*−*8*, *31*−*34*). It was found here that the interaction of both Lys residues is needed for effective formation of the occluded nucleotide state (Figures 4 and 5). This is again consistent with previous studies with the single mutants K429R and K1072R, both of which prevented transition-state formation as measured by Vi-ADP trapping but did not prevent initial MgATP binding as evaluated by 8-azido-ATP photolabeling (*14*).

The occluded nucleotide state in E552A/E1197A Pgp was favored by the presence of drugs (Figures 1 and 2). Earlier work had concluded that one function of the "Q-loop" residues Gln-471 and Gln-1114 in Pgp was to facilitate communication between drug-binding sites and NBDs (*26*). Here, it was seen that mutations E552A/E1197A/Q471A and E552A/E1197A/Q1114A strongly impaired the ability to bind MgADP and MgATP tightly even in the presence of verapamil. This could be explained as being due to the mutations preventing the stimulatory effect of verapamil. However, some caution should be exercised because the single mutations Q471A or Q1114A alone reduced but did not completely prevent the stimulation of ATPase by verapamil or other drugs (*26*).

The work reported above leads us to propose that the catalytic pathway of Pgp does involve the occluded nucleotide/NBD dimer conformation, as depicted in the model of Figure 7. Steps at which drugs, chemical modification reagents, and different mutations are suggested to impact the pathway are shown. The model depicts a mechanism in which (1) loose binding of MgATP at two NBDs, in conjunction with the binding of drug at the drug-binding site, triggers formation of the closed, dimeric NBD conformation in which nucleotide is occluded. One ATP is bound more tightly than the other (bold in the diagram), and we hypothesize that this ATP is committed to hydrolysis. (2) The transition state occurs at the site of this tightly bound nucleotide, and ADP and P_i are formed. (3) P_i and then ADP will be released from this site as the occluded dimer opens, which allows new ATP to bind.

If the nonhydrolyzed ATP that is present in the occluded dimer is retained after release of product ADP (as suggested in the model), then hydrolysis in alternating sites (NBDs) will be operative as originally hypothesized in ref *1*. Such a scenario was discussed and favored in ref *41* on the basis of computer simulation of the NBD dimer interface.

Previous attempts to reverse multidrug resistance conferred by Pgp in cancer have largely derived from the strategy of developing safe drug analogues that compete effectively for occupation of the drug-binding sites in time average and thus allow drugs to escape capture and extrusion by Pgp. The work reported here encourages a different approach, namely, the development of small molecules or peptides that bind in the NBD dimer interface and prevent closure, thus preventing Pgp from accessing the occluded nucleotide conformation and the transition state for ATP hydrolysis.

ACKNOWLEDGMENT

We thank Sarah Lockwood for excellent technical assistance.

REFERENCES

1. Senior, A. E., Al-Shawi, M. K., and Urbatsch, I. L. (1995) The catalytic cycle of P-glycoprotein, *FEBS Lett. 377*, 285−289.
2. Borst, P., and Elferink, R. O. (2002) Mammalian ABC transporters in health and disease, *Annu. Rev. Biochem. 71*, 537−592.
3. Gottesman, M. M., Fojo, T., and Bates, S. E. (2002) Multidrug resistance in cancer: Role of ATP-dependent transporters, *Nat. Rev. Cancer 2*, 48−58.
4. 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.
5. Higgins, C. F., and Linton, K. J. (2004) The ATP switch model for ABC transporters, *Nat. Struct. Mol. Biol. 11*, 918−926.
6. 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.
7. 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.
8. Chen, J., Lu, G., Lin, J., Davidson, A. L., and Quiocho, F. A. (2003) A tweezers-like motion of the ATP-binding cassette dimer in an ABC transport cycle, *Mol. Cell 12*, 651−661.
9. Locher, K. P., Lee, A. T., and Rees, D. C. (2002) The *E. coli* BtuCD structure: A framework for ABC transporter architecture and mechanism, *Science 296*, 1091−1098.
10. Verdon, G., Albers, S. V., van Oosterwijk, N., Dijkstra, B. W., Driessen, A. J. M., and Thunnisen, A. M. W. H. (2003) Formation of the productive ATP−Mg^{2+}-bound dimer of GlcV, an ABC-ATPase from *Sulfolobus solfataricus*, *J. Mol. Biol. 334*, 255−267.

11. Moody, J. E., Millen, L., Binns, D., Hunt, J. F., and Thomas, P. J. (2002) Cooperative, ATP-dependent association of the nucleotide binding cassettes during the catalytic cycle of ATP-binding cassette transporters, *J. Biol. Chem. 277*, 21111−21114.
12. Janas, E., Hofacker, M., Chen, M., Gompf, S., van der Does, C., and Tampé, R. (2003) The ATP hydrolysis cycle of the nucleotide-binding domain of the mitochondrial ATP-binding cassette transporter Mdl1p, *J. Biol. Chem. 278*, 26862−26869.
13. Vergani, P., Lockless, S. W., Nairn, A. C., and Gadsby, D. C. (2005) CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains, *Nature 433*, 876−880.
14. Urbatsch, I. L., Julien, M., Carrier, I., Roussear, M. E., Cayrol, R., and Gros, P. (2000) Mutational analysis of conserved residues in the nucleotide binding sites of P-glycoprotein, *Biochemistry 39*, 14138−14149.
15. Sauna, Z. E., Müller, M., Peng, X. H., and Ambudkar, S. V. (2002) Importance of the conserved Walker B glutamate residues, 556 and 1201, for the completion of the catalytic cycle of ATP hydrolysis by human P-glycoprotein (ABCB1), *Biochemistry 41*, 13989−14000.
16. Orelle, C., Dalmas, O., Gros, P., Di Pietro, A., and Jault, J. M. (2003) The conserved glutamate residue adjacent to the Walker-B motif is the catalytic base for ATP hydrolysis in the ATP-binding cassette transporter BmrA, *J. Biol. Chem. 278*, 47002−47008.
17. 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.
18. Urbatsch, I. L., Gimi, K., Wilke-Mounts, S., Lerner-Marmarosh, N., Rousseau, M.-E., Gros, P., and Senior, A. E. (2001) Cysteines 431 and 1074 are responsible for inhibitory disulfide cross-linking between the two nucleotide-binding sites in human P-glycoprotein, *J. Biol. Chem. 276*, 26980−26987.
19. Loo, T. W. Bartlett, M. C., and Clarke, D. M. (2002) The "LSGGQ" motif in each nucleotide-binding domain of human P-glycoprotein is adjacent to the opposing walker A sequence, *J. Biol. Chem. 277*, 41303−41306.
20. Lee, J. Y., Urbatsch, I. L., Senior, A. E., and Wilkens, S. (2002) Projection structure of P-glycoprotein by electron microscopy. Evidence for a closed conformation of the nucleotide binding domains, *J. Biol. Chem. 277*, 40125−40131.
21. 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.
22. Tombline, G., Bartholomew, L., 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.
23. Tombline, G., Bartholomew, L., Gimi, K., Tyndall, G. A., and Senior, A. E. (2004) Synergy between conserved ABC signature Ser residues in P-glycoprotein catalysis, *J. Biol. Chem. 279*, 5363−5373.
24. Qu, Q., Russell, P. L., and Sharom, F. J. (2003) Stoichiometry and affinity of nucleotide binding to P-glycoprotein during the catalytic cycle, *Biochemistry 42*, 1170−1177.
25. 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.
26. Urbatsch, I. L., Gimi, K., Wilke-Mounts, S., and Senior, A. E. (2000) Investigation of the role of glutamine-471 and glutamine-1114 in the two catalytic sites of P-glycoprotein, *Biochemistry 39*, 11921−11927.
27. Lerner-Marmarosh, N., Gimi, K., Urbatsch, I. U., 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.
28. Beaudet, L., Urbatsch, I. L., and Gros, P. (1998) High-level expression of mouse Mdr3 P-glycoprotein in yeast *Pichia pastoris* and characterization of ATPase activity, *Methods Enzymol. 292*, 397−413.
29. 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.
30. 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.
31. Verdon, G., Albers, S. V., van Oosterwijk, N., Dijkstra, B. W., Driessen, A. J. M., and Thunnisen, A. M. W. H. (2003) Crystal structures of the ATPase subunit of the glucose ABC transporter from *Sulfolobus solfataricus*: Nucleotide-free and nucleotide-bound conformations, *J. Mol. Biol. 330*, 343−358.
32. Gaudet, R., and Wiley, D. C. (2001) Structure of the ABC ATPase domain of human TAP1, the transporter associated with antigen processing, *EMBO. J. 20*, 4964−4972.
33. Karpowich, N., Martsinkevich, O., Millen, L., Yuan, Y. R., Dai, P. L., MacVey, K., Thomas, P. J., and Hunt, J. F. (2001) Crystal structures of the MJ1267 ATP binding cassette reveal an induced-fit effect at the ATPase active site of an ABC transporter, *Structure 9*, 571−586.
34. Yuan, Y. R., Blecker, S., Martsinkevich, O., Millen, L., Thomas, P. J., and Hunt, J. F. (2001) The crystal structure of the MJ0796 ATP-binding cassette. Implications for the structural consequences of ATP hydrolysis in the active site of an ABC transporter, *J. Biol. Chem. 276*, 32313−32321.
35. Sankaran, B., Bhagat, S., and Senior, A. E. (1997) Photoaffinity labelling of P-glycoprotein catalytic sites, *FEBS Lett. 417*, 119−122.
36. Hung, L. W., Wang, I. X., Nikaido, K., Liu, P. Q., Ames, G. F., and Kim, S. H. (1998) Crystal structure of the ATP-binding subunit of an ABC transporter, *Nature 396*, 703−707.
37. 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.
38. Senior, A. E., and Bhagat, S., (1998) P-glycoprotein shows strong catalytic cooperativity between the two nucleotide sites, *Biochemistry 37*, 831−836.
39. Loo, T. W., and Clarke, D. M. (1995) Covalent modification of human P-glycoprotein mutants containing a single cysteine in either nucleotide-binding fold abolishes drug-stimulated ATPase activity, *J. Biol. Chem. 270*, 22957−22961.
40. Georges, E., Zhang, J. T., and Ling, V. (1991) Modulation of ATP and drug binding by monoclonal antibodies against P-glycoprotein, *J. Cell Physiol. 148*, 479−484.
41. 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.
42. Urbatsch, I. L., Sankaran, B., Weber, J., and Senior, A. E. (1995) P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site, *J. Biol. Chem. 270*, 19383−19390.
43. 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.
44. Reyes, C., and Chang, G. (2005) Structure of the ABC transporter MsbA in complex with ADP•vanadate and lipopolysaccharide, *Science 308*, 1028−1031.
45. Senior, A. E., Gros, P., and Urbatsch, I. L. (1998) Residues in P-glycoprotein catalytic sites that react with the inhibitor 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, *Arch. Biochem. Biophys. 357*, 121−125.
46. Szabo, K., Welker, E., Bakos, W., Muller, M., Roninson, I., Varadi, A., and Sarkadi, B. (1998) Drug-stimulated nucleotide trapping in the human multidrug transporter MDR1. Cooperation of the nucleotide binding domains, *J. Biol. Chem. 273*, 10132−10138.
47. Qu, Q., and Sharom, F. J. (2001) FRET analysis indicates that the two ATPase active sites of the P-glycoprotein multidrug transporter are closely associated, *Biochemistry 40*, 1413−1422.

BI0509797