P-glycoprotein Catalytic Mechanism

STUDIES OF THE ADP-VANADATE INHIBITED STATE*

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Kinetics of inhibition of ATPase activity of pure mouse Mdr3 P-glycoprotein upon incubation with MgADP and vanadate were studied along with the trapping of [14C]ADP in presence of vanadate. The presence of verapamil strongly magnified both effects. Inhibition of ATPase was also increased by several other drugs known to bind to drug-binding sites. Inhibition by ADP-vanadate was slow and depended cooperatively on nucleotide binding. Stoichiometry of [14C]ADP trapping by vanadate was 1 mol/mol P-glycoprotein at full inhibition. Site catalytic mutants prevented [14C]ADP trapping, whereas interdomain signal communication mutants reduced it in approximate correlation with their effects upon drug stimulation of ATPase. In explanation of the results, we propose that a "closed conformation" of dimerization and interdigation of the two nucleotide-binding domains is necessary to allow inhibition by ADP-vanadate. The results suggest that such a conformation occurs naturally during ATP hydrolysis. It is proposed that in order for the catalytic transition state to form, the two nucleotide-binding domains dimerize to form an integrated single entity containing two bound ATP with just one of the two ATP being hydrolyzed per dimerization event.

P-glycoprotein (Pgp) is a prominent member of the ABC-transporter family of membrane proteins that uses the energy of ATP hydrolysis to exclude hydrophobic compounds from cells. In human, it was first recognized as a major potential obstacle to successful chemotherapy of cancer because of its expression in cancer cells and was later realized to function physiologically in such strategic locations as the blood-brain barrier, intestine, placenta, and elsewhere as an important agent in protection from environmental and dietary toxins. More recent appreciation of its role in impedig the action of anti-AIDS therapy and its potentially general role in reducing the efficacy of many hydrophobic drugs, new and old, have made it a target of intense investigation. Recent reviews of the biochemistry and pharmacology of Pgp may be found in Refs. 1–6. Our research has focused on the catalytic mechanism by which ATP is hydrolyzed and the energy transduced into drug transport. In 1995, we presented schemes for the hydrolysis of ATP at a single catalytic site (7), also for the interaction of the two ATP-binding sites in catalysis and transduction of the energy of hydrolysis to the drug binding site(s) situated in the membrane bilayer (8). Both ATP-binding sites were shown to have catalytic capability. Release of product P i was thought to occur before the release of ADP, which was rate-limiting. Interaction of the two ATP-binding sites was concluded to be an integral and a necessary facet of catalysis with hydrolysis of ATP occurring sequentially and alternately at each site. Formation and collapse of the transition state of catalysis were postulated as critical events in driving the changes in drug-binding site affinity and orientation (inward versus outward facing) necessary for binding and extrusion of drug with a stoichiometry of one ATP hydrolyzed per change in drug-binding site affinity and orientation. Later studies of Pgp and also of ABC transporter homologs such as bacterial LmrA and the maltose transporter have supported and extended the earlier schemes (9–11). New ideas incorporated into more recent schemes include the concept of reciprocating pairs of drug-binding sites (10) and of the requirement for a second ATP hydrolysis event to "reset" the drug-binding site for drug extrusion (9). Another concept, recently introduced, envisions that the ATP-binding event provides the primary driving force for transport (12). Electron microscopy studies provide evidence for conformational changes in the membrane domains observed upon nucleotide binding (12); however, biochemical evidence has been provided showing that the drug-binding site also changes conformation in response to ATP hydrolysis per se (13, 14).

Recent work (15) demonstrated that, at concentrations of MgATP and MgADP present in the cytoplasm of mammalian cells, both nucleotide-binding domains (NBD) in "resting" Pgp would be expected to bind MgATP (15). Our original scheme for ATP-driven drug transport (8) envisaged that upon attainment of a conformation in which two ATP were bound in the NBDs concurrent with occupation of the drug-binding site, an intimate interaction of the two NBDs would be engendered as a prerequisite for ATP hydrolysis. In later work we provided evidence for the interaction of the two NBDs in Pgp in catalysis and suggested that formation of a single transition state complex involved liganding from catalytic side-chains of both NBDs (16) in what we termed a closed conformation of the catalytic sites. Support for this idea has since come from cross-linking studies showing that catalytic sites of the two NBDs in Pgp can be very close (17–20) from low resolution electron microscopy studies of Pgp (12, 21–22) and from fluorescence resonance energy transfer studies of the two Pgp catalytic sites (23). In other ABC transporters the NBDs also interact with each other, as shown by photocleavage studies of the maltose trans-
Fig. 1. Diagrammatic representation of a proposed mechanism of P-glycoprotein involving dimerization of the nucleotide-binding domains. On the left is the Open form of Pgp. ATP binds loosely to both NBDs ($K_a$(MgATP) = 1 mM (28, 29), $K_a$(MgATP) = 0.4 mM (15)), and at this stage catalytic site side-chains are loosely engaged (see "Discussion" in Ref. 16). High affinity drug binding occurs from the inner membrane leaflet (30–34). When both ATP-binding sites and the drug-binding site are occupied, the Closed form ensues (right). Here both NBDs interdigitate with catalytic side-chains from NBD1 inserting into NBD2 and vice versa. The catalytic transition state forms around one of the two nucleotide γ-phosphates, and collapse of this transition state with P_i release leads to low affinity outward-facing drug binding by interdomain signal communication. Whereas the two ATP molecules are drawn here in "head-to-tail" orientation as in ArsA (35), recent cross-linking experiments would also support a "head-to-tail" orientation in Pgp (19, 20; see also Ref. 25). The depiction here of the two ATP molecules extended normal to the membrane plane is purely for convenient representation.

porter (24) and most recently from x-ray structural characterization of the ABC transporters MJ0796 (25) and BtuCD (26). This x-ray work showed in detail how the two NBD subunits in ABC transporters can interdigitate to form a “dimer interface” at which the nucleotides are bound. Thus, “dimerization” of the NBDs appears now to be a well supported feature. In Fig. 1 we show a proposed mechanism of Pgp incorporating the concept of NBD dimerization. A diagram of this kind encourages the thinking that the catalytically active site is actually a composite entity consisting of the two NBDs and two bound ATP, only one of which will be hydrolyzed during one catalytic event. Movement of the NBDs between open and closed forms (Fig. 1) will occur in synchrony with drug transport. However, it is clear that many features of the Pgp catalytic mechanism remain to be elucidated, such as the nature of interdomain communication between the drug-binding site(s) and the NBDs, the structural and functional character of the ATP binding pockets and the transition state, and the time sequence of the dimerization phenomenon in relation to the progression of catalysis through intermediate species. Earlier, we introduced the approach of vanadate (Vi)-induced trapping of nucleotide as a tool to study the catalytic sites in Pgp (7, 36). Incubation of MgATP with Vi and Pgp was shown to cause strong inhibition of Pgp ATPase brought about by tenacious trapping of MgADP in stoichiometry of just 1 mol ADP/mol Pgp. As was discussed (7, 36), biochemical evidence from other ATPase enzymes, notably myosin and dynein, had led to the idea that the pentacovalent Vi in the trapped MgADP-Vi complex mimicked the transition state structure around the γ-phosphate of MgATP in an associative trigonal bipyramidal transition state structure (37). Smith and Rayment (38) reported an x-ray structure of myosin bound to MgADP-vanadate, which confirmed this geometry. Biochemical and mutagenesis studies of Pgp have supported such an assignment in Pgp (16, 39–42). The rate of onset of Vi-induced inhibition and trapping of nucleotide in the presence of MgATP was shown to be accelerated by drugs (40, 41). Since acceleration of the rate of ATP hydrolysis is a well known property of drugs (43, 44), this provided one explanation of the accelerated inhibition, however, it is not yet clear whether the presence of drugs specifically stabilizes the transition state or not. This question also relates to the broader question of the nature of interdomain communication between drug-binding sites and catalytic sites in Pgp.

We showed earlier that incubation of Pgp with Vi and either MgADP or the analog Mg-8-azido-ADP led to strong inhibition of ATPase and tenacious trapping of MgADP-Vi (or Mg-8-azido-ADP) in catalytic sites (7, 36), this occurring in the absence of any catalytic turnover. Evidence indicated that whether Pgp was initially incubated with MgATP or MgADP together with Vi, the resultant inhibited Pgp-MgADP-Vi showed similar properties, suggesting that the same complex was formed. Inhibition and trapping of the natural MgADP represents therefore an alternative approach to study the effect of drugs on the transition state, which has not so far been studied in detail. In this paper we have utilized pure, soluble, wild-type mouse Mdr3 Pgp to characterize the formation of the MgADP-Vi complex from MgADP and Vi and have additionally studied mutations that impair catalysis directly or that impair interdomain communication between drug sites and catalytic sites. Use of pure protein with the natural MgADP ligand facilitated accurate calculation of stoichiometries and avoids possible limitations inherent from use of photactivated analogs.

EXPERIMENTAL PROCEDURES

Purification of Pgp

Wild-type and mutant mouse Mdr3 Pgp were expressed in Pichia pastoris and purified to homogeneity as described previously (16, 45). Expression and purification of human Mdr1 Pgp were as described previously (17).

Activation of Pgp with DTT and Lipid

Activation of Pgp with DTT and lipid was essentially as described previously (16, 45). Pgp was incubated with DTT (8 mM) and Escherichia 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.

Assay of ATPase Activity

Linked Enzyme Assay—This assay was used routinely unless otherwise stated in the text. This assay utilizing pyruvate kinase and phos-

2 The ABC transporter MsbA did not show such close association of the two NBDs (27). However, use of OsCl4 to improve resolution may well have dictated orientation of the NBDs seen in this structure (Fig. 2 of Ref. 27).
phenoxyphenolpyruvate to regenerate ATP from ADP and lactate dehydrogenase to couple ATP hydrolysis to NADH oxidation by pyruvate was performed at 37°C as described previously (36). MgATP (10 mM) and verapamil (150 μM) were included.

Charcoal Adsorption Assay—This assay was used for K_0.5(ADP) measurement and was performed essentially as described previously (46). Activated Pgp (0.5 μg in 25-μl volume) was added with mixing to 25 μl of 4 mM Tris/Cl, pH 7.5, 0.1 mM EDTA, 1 mM of [γ-32P]ATP, varied concentration of MgATP, and 2 mM excess MgCl_2. Incubation was at 37°C for appropriate times. Reaction was stopped by addition of 0.75-ml ice-cold acid-washed charcoal (catalog C-5510, Sigma), which was suspended at 10% (w/v) in 10 mM EDTA immediately before use. After ≥3-h incubation in ice, samples were centrifuged (14,000 × g for 30 min at 4°C) and the supernatant containing [32P]Pi, was counted by the Cerenkov method. Background samples lacking protein but with all of the other components were subtracted at each MgATP concentration. For determination of K_0.5(ADP), MgADP was included in the assays at 250, 500, and 1000 μM and MgATP concentration was varied from 25 μM to 3 mM. All of the reactions were linear with time, and <10% of the added MgATP was hydrolyzed.

Centrifuge Column Elution of Pgp
1-ml centrifuge columns of Sephadex G-50 in disposable syringes were equilibrated with 50 mM Tris/Cl, pH 7.5, and 0.001% N-dodecyl-β-o-maltoside at 4°C. The pre-spin was at 1000 rpm in a clinical centrifuge for 2 min at 4°C, after which Pgp (10–20 μg in 100 μl) was loaded onto the column and eluted by a second spin. Recovery of Pgp was 70–95% as judged by ATPase and/or protein assays.

Inhibition of Pgp ATPase by ADP plus Vi or by ATP plus Vi
10–20 μg of activated Pgp were preincubated with 200 μM sodium orthovanadate, 1 mM MgCl_2, and 50 mM Tris/Cl, pH 7.5, in 100-μl total volume at 37°C for varied time as described in the text. NaADP or NaATP was present at required concentration as described in the text. Incubations were started by the addition of Pgp and halted by transfer to ice, then passage through centrifuge columns to remove unbound ligand. Stock orthovanadate solutions (100 mM) were prepared from Na_3VO_4 (Fisher Scientific) at pH 10 and boiled for 2 min before each use. Other variations of the conditions are given in the text.

Estimation of Stoichiometry of Vi-trapped Nucleotide
Where MgADP and Vi were used, 100 μM [8-14C]ADP replaced ADP in the preincubation. Where MgATP and Vi were used, [α-32P]ATP was used. Control experiments showed that in absence of Pgp, elution of radioactive nucleotide was negligible. The amount of trapped radioactive ADP was proportional to the amount of Pgp applied in the range 5–20 μg (35–140 pmol). Stoichiometry of trapped nucleotide was calculated using a molecular mass of 142 kDa for Pgp (47).

Reactivation of Pgp ATPase Activity
Pgp was inactivated as above in the presence of Vi plus ADP or Vi plus ATP and then passed through centrifuge columns to remove unbound ligands. Eluates were then incubated at 37°C for various times and then assayed for ATPase by linked enzyme assay.

Routine Procedures
Accurate Pgp concentration was calculated by reference to a standard pure wild-type preparation, which had been subjected to amino acid analysis. A range of amounts of wild-type or mutant Pgp were run on SDS-gels alongside this reference standard, stained with Coomassie Blue, and scanned. For rapid analysis, the Bradford assay was used with bovine serum albumin as standard normalized to the reference Pgp standard. Verapamil and other drugs were added as solution in Me2SO, keeping the final solvent concentration <2% (v/v), and control “no drug” samples contained the same amount of Me2SO. SDS gel electrophoresis was performed as described previously (16).

Materials
NaADP was purchased from Sigma (Catalog A-2754) or Roche Applied Science (296 675). [8-14C]ADP and [α-32P]ATP were from PerkinElmer Life Sciences. E. coli lipids (acetone/ether-precipitated) were from Avanti Polar lipids.

RESULTS
Inhibition of Wild-type Mouse Mdr3 Pgp after Preincubation with Vanadate and MgADP or Vanadate and MgATP—We previously reported that ATP (or 8-azido-ATP) in conjunction with Mg_2+ and Vi could produce long-lived inhibition of Pgp caused by tenacious trapping of the Vi-nucleoside diphosphate complex at a stoichiometry of 1 mol/mol Pgp (7, 36). Similar long-lived inhibition was seen when ADP (or 8-azido-ADP) was substituted for ATP, although the stoichiometry of trapped nucleotide was not established in that case. For this earlier work we used primarily Chinese hamster ovary cell plasma membrane preparations enriched in Pgp. As discussed in Introduction, there is good support for the concept that the Pgp-MgADP-Vi complex mimics the natural transition state of ATP hydrolysis. Subsequently, we and others have shown that purified detergent-soluble mouse (Mdr3) Pgp forms this same complex when incubated with MgATP or Mg-8-azido-ATP (16, 39).

Here we used purified mouse Mdr3 Pgp to investigate the formation of the Pgp-MgADP-Vi complex in the absence of ATP hydrolysis using MgADP as the loading nucleotide. Fig. 2A shows the inhibition of Pgp ATPase activity as a function of

Fig. 2. Inhibition of the ATPase activity of mouse Mdr3 P-glycoprotein upon preincubation with Vi and MgADP or with Vi and MgATP. A, activated Pgp (see “Experimental Procedures”) was preincubated with 100 μM NaADP, 200 μM Vi, 1 mM MgCl_2, and 150 μM verapamil at 37°C for indicated times. Unbound ligands were removed by passage through centrifuge columns, and eluates were assayed for ATPase activity using the linked enzyme assay. The solid line is a fit to the exponential equation as in A, yielding values of a = 88% and b = 1.55 min⁻¹ (first order rate constant = 2.6 × 10⁻² s⁻¹; t1/2 = 27 s). Data points are means of 5–6 experiments.
time in the presence of verapamil. Rate of onset of inhibition was slow with a half-time of 37 min, and approximately 90% inhibition was reached after 120 min. Control experiments showed that inhibition was only seen if the ligands ADP, Mg\textsuperscript{2+}, and Vi were present. An omission of any one prevented inhibition. From the first order rate constant (3.4 × 10\textsuperscript{-4} s\textsuperscript{-1}), an apparent second order rate constant of 3.1 M\textsuperscript{-1} s\textsuperscript{-1} in respect to MgADP was calculated. This is low in comparison to simple protein-ligand association reactions and suggests that a slow protein isomerization step has to occur after initial MgADP binding to allow the Pgp-MgADP-Vi complex to form.\textsuperscript{3}

In previous work using hamster Pgp in plasma membrane preparations, an equivalent half-time of 4.8 min was found for inhibition (36). A possible explanation for the difference could be the different lipid environment of pure Pgp activated with E. coli lipid versus Pgp in mammalian plasma membranes or insufficient lipids or the presence of detergent. We addressed these questions by varying the ratio of lipid to protein during activation of Pgp from 1/1 up to 50/1 by supplementing the E. coli lipids with phosphatidylcholine, phosphatidylserine, and cholesterol, which better mimics mammalian plasma membrane lipid composition (29, 48), and by reconstituting Pgp into proteoliposomes by extensive dialysis to remove detergent. None of these treatments increased the rate of onset of inhibition. Thus, the rate is probably an intrinsic property of the species of Pgp under study.

In contrast, the inhibition of Mdr3 Pgp was much more rapid with Vi and MgATP (Fig. 2B). The half-time was 27 s, which is almost two orders of magnitude faster than with MgADP. For comparison, the half-time for inhibition of hamster Pgp in plasma membranes by Vi plus MgATP was 10 s (36), which is similar to that seen here with Mdr3 Pgp. Hydrolysis of MgATP by Mdr3 Pgp reached 10 s\textsuperscript{-1} at saturating (10 mM) MgATP, and at the concentration of MgATP (100 \mu M) and other conditions used in the inhibition experiments, the measured rate of hydrolysis was 0.38 s\textsuperscript{-1}. A comparison of the rate of hydrolysis with the first order rate constant for inhibition (0.026 s\textsuperscript{-1}) demonstrates that not every turnover event leads to the formation of a trapped and inhibited intermediate, at least under the conditions used here. The calculated apparent second order rate constant for inhibition of 198 M\textsuperscript{-1} s\textsuperscript{-1} in respect to MgATP is consistent with this conclusion.

**Verapamil and a Range of Other Drug Substrates Accelerate the Rate of Inhibition by Vi plus MgATP**—Fig. 3A shows the effect of verapamil on the inhibition of Pgp ATPase activity by Vi plus ADP. Verapamil has been shown earlier to be among the most effective stimulators of Mdr3 Pgp ATPase (49). Little inhibition was seen in the absence of added drug or at verapamil concentrations <1 \mu M. Half-maximal inhibition was seen at 12 \mu M, and maximal inhibition (70% here because a 60-min incubation time was used, see Fig. 2A) was reached at around 150 \mu M verapamil, which is also the concentration at which maximal ATPase is elicited. Kinetic experiments utilizing varied concentrations of verapamil indicated that formation of the inhibited state was faster and more complete with increasing concentrations of verapamil (not shown). The data show that, in the absence of hydrolysis, binding of drug to the drug-binding site greatly enhances the formation of the transition state-like conformation of the catalytic site and indicate that drug binding accelerates the slow isomerization process in the formation of this state and/or shifts the equilibrium toward it by stabilizing the transition state. Fig. 4 demonstrates that a range of other drugs also facilitated Vi plus ADP-induced inhibition of Pgp under nonhydrolysis conditions, buttressing the conclusion.

**Verapamil Stimulation of the Rate of Inhibition in the Presence of Vi plus ATP**—Fig. 3B shows the verapamil dependence of Vi inhibition with MgATP after a 20-min preincubation. A difference from the data of Fig. 3A was that significant inhibition was seen in the absence of drug. Under these conditions activated Mdr3 Pgp showed a low but significant “basal” ATPase activity (basal activity was 0.3 \mu mol/min/mg Pgp or 0.2 s\textsuperscript{-1}, as compared to activity of 4.2 \mu mol/min/mg Pgp, or 9.7 s\textsuperscript{-1} in presence of 150 \mu M verapamil). Inhibition increased with increasing verapamil with half-maximal inhibition seen at 2 \mu M verapamil. The data indicate that verapamil binding favors a conformation leading to inhibition by Vi when hydrolysis is occurring and/or stabilizes the transition state conformation. The concentrations of verapamil required for half-maximal effect are similar whether ADP or ATP are the nucleotide ligands.

Whether inhibition was induced by Vi plus ADP or by Vi plus...
ATP, the rate constant for reactivation after removal of unbound Vi and nucleotide by centrifuge column elution was the same (3.73 x 10^{-4} s^{-1} and 3.61 x 10^{-4} s^{-1}, respectively), indicating that the same transition state-like conformation was formed. Full reactivation was achieved in both cases. The presence or absence of verapamil in the medium during reactivation did not affect the rates of reactivation.

Stoichiometry of Trapped ADP after Inhibition of Pgp by Vi plus ADP—We have previously demonstrated trapping of ADP in stoichiometry of 1 mol/mol Pgp after inhibition by Vi plus ATP (7, 16, 36). Fig. 5 describes the stoichiometry of trapped ADP in the case of inhibition with Vi plus ADP. In presence of Vi and verapamil, the stoichiometry of trapped [14C]ADP was 0.65 mol/mol Pgp after a 60-min incubation and 0.85 mol/mol after a 120-min incubation. The data correspond well with the observed inhibition of 65 and 85%, respectively, seen in this experiment and extrapolate to a stoichiometry of 1.0 [14C]ADP trapped/mol Pgp at full inhibition. The data support previous findings with Vi and ATP that only one NBD can enter the transition state at any one time. Fig. 5 confirms that verapamil has a strong effect on the trapping of ADP, consistent with the data in Fig. 3. The experiment in Fig. 5 was repeated using human MDR1 Pgp instead of mouse protein. The results (not shown) were very similar to those of Fig. 5 with the human Pgp showing strong verapamil dependence of [14C]ADP trapping. It was important to establish that the inhibition seen in presence of Vi plus ADP was not caused, even partly, by traces of ATP present in commercial samples of ADP. According to the manufacturers’ analyses, the samples of ADP purchased contained <0.05% ATP (Sigma, Roche Applied Science, and PerkinElmer Life Sciences). Thus, potentially, using 100 μM ADP (as in Figs. 2–5) up to 0.05 μM ATP might be present and could confound interpretations. The experiment in Fig. 6 was performed to put these fears to rest. Inhibition by Vi plus ADP was carried out using nonradioactive ADP spiked with 1 or 5 μM [α-32P]ATP. Full (90%) inhibition of ATPase was seen in all of the samples. With 50 μM [α-32P]ATP alone, the stoichiometry of trapped [32P] was close to 1 mol/mol Pgp, and at lower concentration of [α-32P]ATP alone the stoichiometry was 0.16 mol/mol (5 μM) or 0.03 mol/mol (1 μM). In the presence of 100 μM ADP these latter values were further reduced to 0.11 and 0.02, respectively. Since even the lowest concentration of [α-32P]ATP used (1 μM) is still 20-fold higher than the maximum contaminating concentration of ATP expected in the ADP, this experiment establishes that the entire inhibitory effect in Figs. 2–5 is the result of trapping of added ADP.

Effects of ADP Concentration on Formation of the Inhibited State Induced by Vi plus ADP—Fig. 7A shows inhibition of ATPase activity as a function of ADP concentration in the presence of Vi. Concentration required for half-maximal inhibition was 28 μM under these conditions. The solid line was a best fit, giving a Hill coefficient of 1.3. The dotted line is a fit with Hill coefficient set to 1.0. Fig. 7B shows similar data obtained by varying the ATP concentration when Pgp was inhibited by Vi plus ATP. Half-maximal inhibition was ob-
to conclude that Gln-471 and Gln-1114 are involved in interdomain signal communication between drug-binding and catalytic sites (50). Here we studied both types of mutant to discern their effect on Vi-induced trapping of ADP in the absence of hydrolysis.

Fig. 8 shows Vi-induced trapping of ADP by “catalytic site mutants” (S430A, S1073A, D551N, and D1196N) in presence of verapamil. They failed to trap any ADP. This observation indicated that they were unable to form the transition state conformation with Vi and added ADP. Fig. 9 shows the results obtained with the “interdomain signal communication mutants.” Q471A and Q1114A mutant Pgp trapped only low amount of ADP, indicating a reduced ability to form the transition state conformation from Vi plus ADP. It was previously shown that ATPase in these mutants was maximally stimulated by only 3.0- and 4.4-fold, respectively, by verapamil, compared with 13-fold in wild type (50). Q471E and Q1114E mutants trapped considerably more ADP, although less than wild type. ATPase activity of these mutants is maximally stimulated by 11- and 9-fold by verapamil. Thus the mutants that showed the greater stimulation of ATPase by verapamil also showed greater tendency to trap ADP when incubated with Vi plus ADP and verapamil, indicating the need for interdomain communication between drug-binding sites and catalytic sites.

Effects of Drug on the Release of ADP from Catalytic Sites—As noted in Introduction, the catalytic scheme for hydrolysis at a single catalytic site that we developed earlier indicates ADP as the rate-limiting step. Two experiments were carried out to determine whether verapamil affected the rate of ADP release from catalytic sites. First, $K_i$(MgADP) against MgATP hydrolysis was measured in the absence or presence of 150 $\mu$M verapamil. MgADP behaved as a classical competitive inhibitor in both situations with $K_i = 140 \mu$M in the absence of verapamil and 263 $\mu$M in its presence. Thus, by this measure, binding affinity for MgADP was essentially the same whether drug was present or not. Given that verapamil accelerates hydrolysis by at least an order of magnitude, it is therefore unlikely that it does so by accelerating ADP release. Second, the rate of release of trapped radioactive [14C]ADP from cata-

**Fig. 7.** Inhibition of ATPase activity as a function of nucleotide concentration. A, activated Pgp was preincubated for 60 min at 37 °C with varied concentration of ADP as indicated and 1 mM MgCl2 in presence of Vi (200 $\mu$M) and verapamil (150 $\mu$M). Unbound ligands were removed by centrifuge column elution, and eluates were assayed for ATPase activity. The dotted line is a fit to the Michaelis-Menten equation (Hill coefficient = 1.0). The solid line is a best fit to the Hill equation with $n = 1.3$. B, the same experiment was performed with ATP as the nucleotide instead of ADP using a preincubation time of 20 min. The dashed line is a fit to the Michaelis-Menten equation (Hill coefficient = 1.0). The solid line is a best fit to the Hill equation with $n = 1.7$. Data points are means of four experiments.

**Fig. 8.** Vi-induced trapping of ADP by catalytic site mutant Pgp. Activated Pgp was preincubated for 120 min at 37 °C with 100 $\mu$M [8-14C]ADP and 1 mM MgCl2 in presence of Vi (200 $\mu$M) and verapamil (150 $\mu$M). Unbound ligands were removed by centrifuge column elution, and eluates were assayed for radioactivity. The mutations were S430A and S1073A in the two Walker A sequences (16) and D551N and D1196N in the two Walker B sequences (39). Data are means of 2–4 experiments.

**Effects of Mutations on Trapping of ADP after Preincubation of Pgp in Presence of Vi and ADP**—We have previously characterized two separate types of mutation in Pgp. One type is characterized by apparent complete loss of ability to hydrolyze ATP and inability to trap nucleotide after incubation with Vi plus ATP. This type is exemplified by the mutations S430A and S1073A in the catalytic site Walker A sequences (16) and D551N and D1196N in the Walker B sequence (39). These mutations affect residues, which are totally conserved in ABC transporters, and are thought to be ligands to the bound Mg nucleotide and transition state in catalytic sites. The second type is exemplified by the mutations Q471A,E and Q1114A,E. Mutagenesis of this invariant glutamine yielded Pgp with partial basal ATPase activity (~10% wild type), which was notably less responsive to drug stimulation than wild-type, leading us
lytic sites after Vi-induced trapping was measured in the presence and absence of verapamil. [14C]ADP was trapped by incubation with Vi as shown in Fig. 7, passed through centrifuge columns to remove unbound ligands, and then incubated in buffer at 37 °C, and samples passed through centrifuge columns to measure residual bound [14C]ADP at the appropriate times. It was found that neither the presence of 150 μM verapamil nor presence of 10 mM MgATP affected the rate of release.

**DISCUSSION**

We used pure, detergent-soluble mouse Mdr3 P-glycoprotein activated by lipid and DTT to study aspects of P-glycoprotein catalytic mechanism with particular emphasis on the formation of the inhibited state by trapping of Vi and ADP to form the ADP-Vi complex at a stoichiometry of 1 mol/mol Pgp. As noted in the Introduction, recent biochemical and structural work on Pgp and other ABC transporters has led to the concept of dimerization of the NBDs as a component of the catalytic mechanism, and we have attempted in our interpretations below to relate this dimerization to the enzymatic mechanism. In the first experiments (Figs. 2A, 3A, 4, and 5), we found that the ADP-Vi inhibited state formed slowly in the absence of ATP hydrolysis when Pgp was incubated with Vi and ADP and that verapamil and other drugs greatly accelerated the inhibition of ATPase and trapping of ADP. Scheme 1 can be envisaged to describe this effect.

\[
\text{Pgp} + \text{MgADP} \rightarrow \text{Pgp-MgADP} \rightarrow \text{Pgp-MgADP-Vi}
\]

**Scheme 1**

In this scheme, binding of MgADP in step one occurs rapidly with a second order rate constant of around 10^6 M\(^{-1}\) s\(^{-1}\) (see footnote 3) and is followed by a slow isomerization step (step 2) with a high activation energy to give a conformation Pgp-MgADP\(^*\), which resembles the transition state much more closely than Pgp-MgADP. Vi can bind to Pgp-MgADP\(^*\) forming strong ligands to the protein side-chains and bound MgADP, thus tenaciously trapping the nucleotide (step 3). We propose that step 2 involves dimerization of the NBDs, forming Closed conformation from Open conformation as discussed in the Introduction and shown in Fig. 1. We envisage that in step 1 binding of MgADP occurs to the two individual NBDs, step 2 is the dimerization step, and Vi binds to the closed conformation (dimerized NBDs) where the transition state normally occurs. There is cooperativity in the formation of the inhibited state (Fig. 7A), suggesting the requirement for binding of ADP to two NBDs as a prerequisite to allow step 2 to occur. Binding of drug apparently lowers the activation energy barrier for dimerization of the NBDs. Dissociation of Vi and ADP, on the other hand, was not affected by drug. Opening of the ADP-Vi-trapped site probably does not occur through the reversal of step 3, therefore.

Inhibition by trapping of ADP-Vi when ATP hydrolysis is occurring can be described by Scheme 2.

\[
Pgp + \text{MgATP} \leftrightarrow \text{Pgp-MgATP} \rightarrow \text{Pgp-MgADP-Pi}
\]

\[
\rightarrow \text{Pgp-MgADP}\rightarrow \text{Pgp-MgADP-Vi}
\]

**Scheme 2**

Steps 1–3 are part of the normal pathway of ATP hydrolysis. In step 1, MgATP binds to both NBDs in the open conformation of Fig. 1. Following the proposal in Fig. 1, dimerization of the NBDs (step 2) occurs to allow the transition state to form in the closed conformation and produce Pgp-MgADP-Pi. This step is strongly cooperative in respect to ATP binding (Fig. 7B), indicating that both NBDs must be occupied to allow dimerization. The presence of drug bound in the drug-binding site presumably lowers the activation energy barrier for dimerization, hence accelerating ATPase and ADP-Vi trapping. Release of P(3) (step 3) occurs before the NBDs move apart, producing Pgp-MgADP\(^*\) in the closed dimeric state. As above, Vi can bind to this form to trap ADP (step 4). In the normal ATPase pathway, the sequence would be as shown in Scheme 3.

\[
Pgp-MgADP\rightarrow Pgp-MgADP\rightarrow Pgp + \text{MgADP}
\]

**Scheme 3**

We postulate that step 5 involves monomerization of the NBDs, thus step 6 (release of ADP) can then occur at a single NBD as envisaged in the alternating sites model (8). As shown under “Results,” not every hydrolysis turnover resulted in Vi inhibition in our experiments, thus under our conditions step 5 was fast enough to allow monomerization of the NBDs, such that productive collisions between Vi and Pgp-MgADP\(^*\) could occur only part of the time.

Cooperativity between the NBDs was stronger with MgADP (Fig. 7B) than with MgATP (Fig. 7A). This is consistent with the more rapid rate of inhibition in presence of MgATP and Vi (Fig. 2B) than in presence of Vi and ADP (Fig. 2A) and supports the idea that dimerization of the NBDs is correlated with inhibition. Recent measurements of nucleotide binding are in accord with these ideas, as it was shown that MgATP and MgADP each bind to two sites in hamster Pgp with similar \(K_d\) values (15). According to the alternating sites mechanism, in
cells the two sites would normally bind ATP before entering the transition state. One interesting question that arises is how does the enzyme determine which of the two ATP in the closed dimeric conformation will actually be hydrolyzed? An alternating sites mechanism requires that the enzyme retains a "conformational memory" of which NBD last hydrolyzed ATP. In the alternating sites mechanism as proposed previously (8), one of the NBDs does retain ATP after the hydrolytic event and this may "mark" that NBD in some way as the next site of hydrolysis.

Drug-binding sites have been reported to occur in high affinity and low affinity states in Pgp (10, 34, 51, 52). The half-maximal concentration of verapamil found here to accelerate ADP-Vi-induced inhibition of ATPase activity (Fig. 3A) was 12 μM, from which it appears that the effect was caused by verapamil binding to a high affinity site. In respect to the effect of verapamil to stimulate the formation of the transition state-like conformation, we note that a similar effect was documented in the maltose transport system. Binding of periplasmic maltose-binding protein complexed with maltose to the membranous MalGFK2 transporter stimulated Vi-trapping of ADP upon incubation with Vi plus MgATP (53).

Our experiments with catalytic site mutants carrying mutations in the Walker A and Walker B sequences support the ideas developed above. These mutations had been shown previously to totally prevent ATP hydrolysis. They also prevent trapping of ADP-Vi in experiments where MgATP was the nucleotide added with Vi (16, 39). Here we show (Fig. 8) that catalytic site mutations placed in either NBD prevent inhibition and trapping of ADP under conditions where hydrolysis is not occurring. The explanation that we offer for this is that the closed conformation in which the catalytic transition state normally forms requires dimerization of the NBDs and that generation of this conformation requires two bound ATP and two fully intact NBDs, each with critical catalytic side-chains interdigitating into the other (trans) NBD, as proposed in Fig. 1. As we argue above, when inhibition is brought about from Vi and MgATP in the medium, dimerization of the NBDs is required to allow the formation of the species Pgp-MgADP* with which Vi reacts. Thus any mutation that prevents formation of the fully correct closed conformation will prevent trapping of ADP-Vi even in the absence of catalytic turnover.

The experiments with interdomain signal communication mutants in Fig. 9 are also supportive of the ideas presented above. We had previously shown (50) that the conserved Gln residues at positions 431 and 1114 of mouse Mdr3 Pgp were involved in interdomain signaling between the drug-binding sites and the catalytic sites. This conclusion has also been drawn from x-ray crystallography studies of ABC transporter NBD domains (54). Here we showed that the effect of verapamil to accelerate trapping of ADP in the presence of Vi was impaired by mutation of the conserved Gln residues to Ala or Glu and the impairment correlated with their impairment of drug stimulation of ATPase activity.

Finally, we should note that the experimental data reported here are in apparent disagreement with a report by Sauna et al. (55). These workers reported that trapping of 8-azido-ADP by Vi in human Pgp (with 8-azido-ADP as the loading nucleotide) was strongly reduced by verapamil. Human Pgp was used in that study, but as we report here, in our work the trapping of ADP by Vi in human Pgp was increased by verapamil just as it was in mouse Pgp. As well as the use of the analog 8-azido-ATP, there are other differences in procedures. However, the explanation for the different results is not obvious. Sauna et al. (56) also reported that the Arrhenius activation energy for Vi trapping was 2.5-fold higher when 8-azido-ADP was the loading nucleotide as opposed to 8-azido-ATP (152 versus 62 kJ/mol). This finding is consistent with the slower rate of trapping seen here with ADP as compared to ATP and supportive of the explanation offered above that trapping with ADP involves a slow protein isomerization step.

In summary, in this work we studied the inhibition of pure mouse Mdr3 Pgp by Vi and MgADP and the trapping of ADP-Vi. Both were stimulated by drugs. Inhibition depended cooperatively on nucleotide binding. We advance the hypothesis that requirement for a closed conformation of Pgp involving dimerization of the two NBDs explains the results and that such a conformation occurs normally during ATP hydrolysis. We propose that the two NBDs must dimerize and interdigitate to form an integrated single entity containing two bound ATPs in order that the catalytic transition state can occur and that just one of the two bound ATP is hydrolyzed per dimerization event. Experiments reported with catalytic site mutants and interdomain signal communication mutants were supportive of this hypothesis.

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