Drug Binding and Nucleotide Hydrolyzability Are Essential Requirements in the Vanadate-Induced Inhibition of the Human P-Glycoprotein ATPase†

U. Subrahmanyeswara Rao*

Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

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ABSTRACT: P-glycoprotein (Pgp) mediates drug transport utilizing the energy released from ATP hydrolysis. However, the mechanism by which Pgp couples these two reactions remains unclear. The present work is undertaken to describe kinetically the first step, which is the interdependence of nucleotide and drug binding to the Pgp by the use of vanadate. Preincubation of human Pgp expressed in Sf9 insect cells with vanadate in the presence of Mg^{2+}, ATP, and verapamil resulted in nearly complete and stable inhibition of the drug-stimulated ATPase function. In contrast, the Pgp ATPase function was nearly unaffected when Mg^{2+}, ATP, or verapamil was omitted. Inhibition was highly specific for divalent cations that support ATP hydrolysis, for nucleotides that serve as substrates of hydrolysis, and for those drugs/compounds that interact with the drug-binding/transport sites of the Pgp. Kinetic analysis indicated that vanadate inhibition was MgATP concentration-dependent with an apparent K_i value similar to the apparent K_m, suggesting that MgATP was bound to a similar ATP-binding site in both the ATPase inhibition and activation reactions. In support of this conclusion, vanadate, in the presence of Mg^{2+} and verapamil, caused selective trapping of 8-azido [α-^{32}P] ATP and covalent labeling of ATP-binding site in the Pgp. Differences were observed in the vanadate-induced inhibition of wild-type and Val^{185} mutant Pgp's with different drug/compounds. These results suggested that the affinity of the interacting drug/compound is a constant and influences the overall stability of the inhibited Pgp species. Possible implications of these observations for the coupling of ATP hydrolysis to drug transport are discussed.

Tumor cells selected in vitro against any single antineoplastic drug often develop cross-resistance to a wide variety of structurally unrelated drugs, and this phenomenon is known as multidrug resistance (MDR)[1] (*1*). These MDR cells frequently express large quantities of a 130−170 kDa plasma membrane glycoprotein, referred to as Pgp (*2*−*5*). It is established that Pgp from a variety of species acts as an energy-dependent transporter that extrudes from cells a spectrum of drugs and compounds with diverse structures (*5*−*7*). The human Pgp is a 1280-amino-acid-long polypeptide and shares considerable sequence and structural homology to the members of ATP-binding cassette transporter superfamily (*3*, *8*). Pgp, a tandemly duplicated molecule, is predicted to have a total of 12 highly hydrophobic transmembrane segments and two cytoplasmically located ATP-binding domains. It is also established that Pgp possesses ATPase function, which is greatly stimulated by numerous anticancer drugs and chemosensitizers (*9*−*11*). In vivo studies carried out in several laboratories have demonstrated that drugs that stimulate Pgp ATPase activity are transported by Pgp, and mutations in the cytoplasmic and transmembrane regions affect both the ATPase and drug efflux functions of the Pgp (*12*−*16*). Works from the laboratories of Roninson (*4*) and Gottesman (*17*) have shown that cells expressing a mutant Pgp containing glycine to valine substitution at amino acid 185 (Val^{185} Pgp) exhibit preferentially increased resistance to colchicine. The increased resistance is due to increased colchicine efflux by Pgp from these cells. Subsequent biochemical studies have demonstrated that Val^{185} Pgp also exhibits preferentially increased affinity for colchicine and colchicine-stimulatable Pgp ATPase activity (*18*). The predominant view from all the above studies is that the ATPase and drug efflux are interrelated functions of the Pgp.

Utilizing the ATPase measurements, we have recently differentiated the drug interactions with Pgp into two types based on the effects of the interacting drug on the ATPase function (*29*). One type of interaction involves binding of the drug to the drug-binding/transport site of the Pgp with concomitant stimulation of ATP hydrolysis. Compounds including verapamil, colchicine, vincristine, and vinblastine interact with the Pgp in this manner and are predicted to be transported by Pgp utilizing the energy released from ATP hydrolysis. On the other hand, certain compounds that include cyclosporins although bind to the drug-binding/transport site of the Pgp with high affinity, they do not stimulate ATP hydrolysis and thus are not transported by the Pgp. Direct demonstration of ATP hydrolysis-dependent drug transport into the isolated membrane vesicles has been difficult owing to the highly hydrophobic nature of these compounds which tend to partition into the lipid bilayer.

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* Address of correspondence: U. S. Rao, Ph.D., Department of Biochemistry and Molecular Biology, Campus Box 984525, University of Nebraska Medical Center, Omaha, NE 68198-4525. Phone: 402-559-6654. Fax: 402-559-6650. E-mail: usrao@unmc.edu.

[1] Abbreviations: MDR, multidrug resistance; Pgp, P-glycoprotein; Sf9 insect cells, *Spodoptera frugiperda* insect cells; P_i, inorganic phosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride).

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Thus, because of the ease with which the ATPase measurements can be carried out and because the drug transport and ATPase activity are integral reactions of the Pgp, several laboratories including ours employ the drug-stimulated ATPase measurements to describe the overall transport cycle of the Pgp (*9*−*11*, *18*, *29*). However, the mechanism by which Pgp hydrolyzes ATP and couples the released energy to the drug transport remains unclear.

A logical approach to understand the energy-coupled drug transport is to first identify the number and location of nucleotide and drug-binding sites in the Pgp molecule. There have been a number of studies attempting to identify covalently modifying nucleotide analogues (*19*), inhibitors, and affinity analogues that bind to the drug-binding/transport sites (*20*−*23*). However, it is not often a simple, direct procedure to use these photoaffinity analogues for labeling the ligand-binding sites, as they tend to nonspecifically label reactive functional groups unrelated to the ligand-binding sites in the protein. Furthermore, the major impediment in identifying the drug-binding sites in the Pgp molecule is that nearly all of the transport substrates identified thus far are highly hydrophobic and partition into the hydrophobic regions of the molecule embedded in the membrane bilayer. Whether such regions represent true drug-binding sites of the Pgp molecule is unclear at present. An alternative approach toward ameliorating these problems is the discovery that, vanadate, a phosphate analogue and potent inhibitor of several ATPases, selectively traps the nucleotides in the myosin ATPase catalytic site and stably inhibits the enzyme activity (*24*, *25*). Employing a similar approach, Senior and co-workers have recently demonstrated that vanadate also stably inhibits the Chinese hamster Pgp ATPase by trapping MgADP in the catalytic site (*25*). However, the role of transport substrate and selectivity of the ligands in the vanadate-induced inhibition of Pgp are not clear. The present study seeks to demonstrate the interdependence of interactions of ligands that participate in the ATP hydrolysis and drug-transport reactions in the vanadate-induced inhibition of Pgp.

MATERIALS AND METHODS

Recombinant Baculoviruses Expressing the Human Pgp's. Preparation of recombinant baculoviruses carrying the human wild-type MDR1 cDNA and characterization of two isolates, MDR1 BV 9 and MDR1 BV 10, were reported previously (*18*). The Pgp's expressed by both of these recombinant baculoviruses were functionally identical, and isolate MDR1 BV 9 was used in the present study as a source of human wild-type Pgp. Preparation of recombinant baculovirus carrying the mutant Val^{185} MDR1 cDNA, termed Val^{185} MDR1 BV, was previously reported (*26*). The Pgp derived from this cDNA is called Val^{185} Pgp in this paper.

Preparation of Membrane Fraction from Sf9 Insect Cells Infected with Recombinant Baculoviruses. Cultured Sf9 insect cells were grown and infected with the recombinant baculoviruses, *Escherichia coli* β-galactosidase cDNA containing baculovirus (β−Gal BV), MDR1 BV 9, and Val^{185} MDR1 BVs, as described previously (*18*). The total membrane fraction from the infected Sf9 insect cells was prepared, resuspended in a glycerol buffer (50 mM Tris, 50 mM mannitol, 2 mM EGTA, 2 mM 2-mercaptoethanol, and 30% (w/v) glycerol, pH adjusted to 7.0 with HCl), and used immediately or stored at −20 °C for later use.

Pgp ATPase Activity Determination. The Pgp ATPase activity was determined by incubating 5 μL of membrane suspension in 0.1 mL of reaction mixture at 37 °C for 10 min. The reaction mixture contained 50 mM Tris, pH 6.8, with HCl, 2 mM EGTA, 2 mM DTT, 50 mM KCl, 5 mM NaN_3, 5 mM $MgSO_4$, 5 mM ATP, and 1 μL of drug prepared in DMSO. The assay was stopped by the addition of 0.1 mL of 5% SDS solution, and the liberated P_i was measured colorimetrically (*27*). Each of the experiments described in this paper was carried out at least 4 times using membranes prepared from more than 5 different batches of infected Sf9 insect cells with essentially the same results.

Vanadate Inhibition of the Pgp ATPase. A colorless stock solution of 10 mM sodium orthovanadate (Sigma) was prepared in distilled water, and its pH was adjusted to 7.0 with MOPS and kept at room temperature for at least 2 weeks before being used in these studies (*25*, *28*).

Membranes (~100 μg of membrane protein) were incubated at room temperature for 5 min in 0.2 mL of 50 mM Tris Cl buffer, pH 7.0, containing 0.1 mM vanadate and other ligands such as Mg^{2+}, ATP, and drugs/compounds as indicated in each individual experiment. The excess ligands present in the preincubation mixtures were removed in the following way. The incubation mixtures were centrifuged at 13 000 rpm in an Eppendorf microfuge for 4 min at 4 °C. The pelleted membranes were resuspended in 0.5 mL of ice cold 50 mM Tris Cl buffer, pH 7.0, and centrifuged as above. The pelleted membranes were then resuspended in a small volume of glycerol buffer and assayed for the verapamil-stimulated ATPase activity as described above. In a typical experiment, the duration of the entire process from the incubation of membranes with ligands to the initiation of ATPase measurements was approximately 30 min. The recovery of the membrane protein in this procedure was consistently greater than 80% of the initial amounts.

Trapping of 8-Azido [α-^{32}P] ATP in the Nucleotide-Binding Site. Membranes (25 μg of membrane protein) were incubated in 0.05 mL of 50 mM Tris Cl buffer, pH 7.0, containing 10 μM (5 μCi) 8-azido[α-^{32}P]ATP, 1 mM $MgSO_4$, 100 μM vanadate, and 50 μM verapamil at room temperature for 5 min. Variation in the composition of preincubation mixtures was indicated in the Figure 4 legend. The incubation mixture was centrifuged at 13 000 rpm in an Eppendorf microfuge for 4 min at 4 °C. The pelleted membranes were resuspended in 0.5 mL of ice cold 50 mM Tris Cl buffer, pH 7.0, and centrifuged as above. The pelleted membranes were resuspended in 10 μL of ice cold 50 mM Tris Cl buffer, pH 7.0, and exposed to 254 nm of radiation using a hand-held ultraviolet light (UVP Products, CA) for 10 min. The samples were then treated with equal volumes of double-strength Laemmli disagregation buffer and run on the SDS−PAGE followed by electroblotting onto PVDF membranes (*18*). The blotted PVDF membrane was first exposed to the phosphorimager screen and the ^{32}P-labeled proteins were detected using Storm 840 Phosphorimager (Molecular Dynamics). The blots were then immunostained using the Pgp-specific C219 antibody in conjunction with horseradish peroxidase-conjugated secondary antibody to localize the Pgp. The peroxidase-labeled blot was developed by the

enhanced chemiluminescence method, using the Amersham ECL kit (*18*).

Other Methods. SDS−PAGE and immunoblotting of proteins were carried out as previously described (*18*). Protein in the membrane suspensions was determined by Coomassie Plus Protein Assay Reagent (Pierce, IL) according to the manufacturer's instructions, using bovine serum albumin as a standard.

Materials. Sodium orthovanadate, ATP for the ATPase assays, β,γ-methylene ATP, verapamil, vinblastine, colchicine, doxorubicin, rhodamine123, 5-fluorouracil, methotrexate, and ouabain were obtained from Sigma. Cyclosporin A was from Sandoz Research Institute (Hanover, NJ). ATP, ADP, GTP, GDP, UTP, UDP, CTP, and 2′-deoxynucleotides used in the inhibition studies were obtained from Pharmacia and Boehringer-Manneheim. 8-Azido[α-^{32}P] ATP was obtained from Andotech, CA. Sf9 culture media were from the Lineberger Cancer Center Tissue Culture Facility at the University of North Carolina, Chapel Hill.

RESULTS

Conditions for Stable Inhibition of the Pgp ATPase Activity. Initially, experiments were carried out to explore the effects of preincubation of Pgp with vanadate in the presence of various catalytic cycle participants on the drug-stimulated ATPase function. Membranes containing the wild-type Pgp were preincubated in a 50 mM Tris buffer, pH 7.0, containing 100 μM vanadate, 10 mM $MgSO_4$, 10 mM ATP, and 50 μM verapamil for 5 min, the excess ligands were removed, and the verapamil-stimulated ATPase activity in the resulting membranes was then determined as described under Materials and Methods. The results indicated that nearly 85% of the original Pgp ATPase activity was inhibited (data not shown). However, when any one of the 3 ligands, that is, Mg^{2+}, ATP, and verapamil, was omitted from the above preincubation mixture, the Pgp ATPase activity was not inhibited by vanadate. Thus, although vanadate is a potent inhibitor of the Pgp ATPase activity, the above observations suggested that stable inhibition of the Pgp ATPase requires the presence of Mg^{2+}, ATP, and verapamil.

I have measured the residual verapamil-stimulated Pgp ATPase activity after the membranes containing wild-type Pgp were preincubated with 10 mM MgATP, 50 μM verapamil, and increasing concentrations of vanadate and washed as described under Materials and Methods. The results presented in Figure 1 indicate that vanadate inhibits the Pgp ATPase activity in a concentration-dependent manner, with an apparent K_i (50% inhibition) value of 10 μM. Pretreatment of Pgp with 100 μM vanadate alone or, in the presence of 10 mM MgATP did not inhibit the drug-stimulated ATPase activity. In other experiments, we have also measured the verapamil-stimulated Pgp ATPase activity in the presence of increasing concentrations of vanadate and obtained a strikingly similar profile of Pgp ATPase inhibition, with 50% inhibition occurring at 7.5 μM vanadate (data not shown). Thus, similar vanadate inhibition profiles and K_i values in these two reactions, that is, inhibition of Pgp ATPase in the preincubations and in the ATP hydrolysis measurements, suggest that vanadate binds to a similar binding site in the Pgp molecule in both of these experimental procedures.

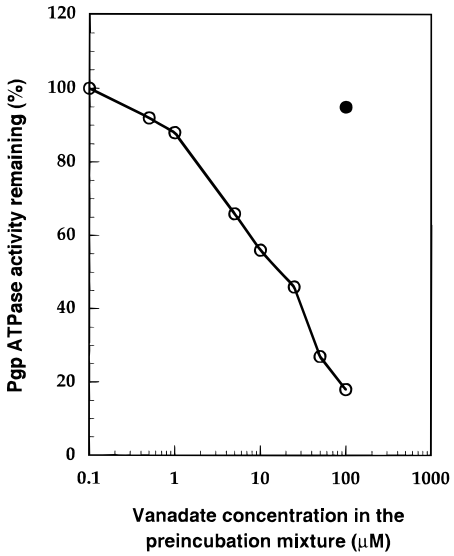

FIGURE 1: Vanadate concentration dependence of Pgp ATPase inhibition. Membranes containing wild-type Pgp (○) were incubated in a 50 mM Tris HCl buffer, pH 7.0, containing 10 mM MgATP, 50 μM verapamil, and increasing concentrations of sodium orthovanadate for 5 min, pelleted by centrifugation, and washed, and the verapamil-stimulated Pgp ATPase activity was determined as described under Materials and Methods. For reasons of clarity, only the ATPase activity of Pgp membranes pretreated with 10 mM MgATP and 100 μM vanadate was shown (●).

To further characterize the role of Mg^{2+}, ATP, and verapamil in vanadate-induced stable inhibition of Pgp ATPase, these ligands were replaced with analogues, their effects were studied, and the results are summarized below.

(i) Metal Ion Requirement in the Vanadate-Induced Pgp ATPase Inhibition. The Mg^{2+} cation in the preincubation mixtures in which the Pgp ATPase activity was strongly inhibited by vanadate, that is, the preincubation mixtures containing the ligands, Mg^{2+}, ATP, and verapamil, was replaced with a variety of metal ions and the effects on verapamil-stimulated ATPase activity was measured (Table 1, upper panel). No Pgp ATPase inhibition was observed in the absence of Mg^{2+}, or in the presence of added Na^+. However, when Mg^{2+}, Mn^{2+}, or Co^{2+} was included in the preincubation mixtures, the ATPase activity was inhibited by approximately 85%, 50%, and 20%, respectively. In other experiments, it was found that verapamil-stimulated Pgp ATPase activity was dependent on a divalent cation and the activities obtained in the presence of Mn^{2+} and Co^{2+} were 50% and 20% of the maximal activity observed in the presence of Mg^{2+} (data not shown). Thus, the divalent metal ions that participate in the Pgp ATPase activation reaction were able to bring about vanadate-induced stable inhibition to a similar extent. These observations suggest that these metal ions are likely bound to the same binding site in the Pgp molecule in both the ATPase activation and the inhibition reactions.

(ii) Nucleotide Requirement in the Vanadate-Induced Pgp ATPase Inhibition. To determine the role of nucleotide on the vanadate-induced Pgp ATPase inhibition, the membranes containing wild-type Pgp were incubated for 5 min in a 50 mM Tris Cl buffer, pH 7.0, containing 10 mM Mg^{2+}, 0.1 mM vanadate, 50 μM verapamil, and a variety of nucleotides (10 mM). The membranes were washed, and the verapamil-stimulated ATPase activities in the resulting membranes were

Table 1: Effect of Different Cations and Nucleotides on the Vanadate-Induced Pgp ATPase Inhibition

ligand	inhibition of Pgp ATPase activity (%)
cation[a]	
none	0
Na^+	<1
Mg^{2+}	84 ± 3
Mn^{2+}	50
Co^{2+}	18
nucleotide[a]	
ATP	84 ± 3
GTP	0
UTP	0
CTP	0
β,γ-methylene ATP	0
2′deoxy ATP	21 ± 4
2′deoxy GTP	0
2′deoxy TTP	0
2′deoxy CTP	0
GDP	<1
UDP	<1
ADP	12 ± 4
ADP + P_i	11 ± 3

[a] In a typical preincubation experiment, wild-type Pgp-containing membranes were incubated in a 50 mM Tris Cl buffer, pH 7.0, containing 10 mM ATP, 10 mM $MgSO_4$, 0.1 mM vanadate, and 50 μM verapamil. In determining the selectivity of cations and nucleotides, the $MgSO_4$ and ATP in the preincubation mixtures were replaced with an equimolar concentration of indicated cations (10 mM) and nucleotides (10 mM), respectively. The P_i concentration was 10 mM. The residual verapamil-stimulated ATPase activity was determined after washing membranes as described under Materials and Methods. One-hundred percent activity is equivalent to the Pgp ATPase activity in 1 mg of membrane protein which was approximately 100 nmol/min. The values are the mean ± standard deviations of 5 experimental results.

then measured as described under Materials and Methods. The lower panel in Table 1 shows that, in the presence of ATP, nearly 85% of the Pgp ATPase activity was inhibited. When the nucleotide in the preincubation mixtures was GTP, UTP, CTP, or β,γ-methylene ATP, no Pgp ATPase activity was inhibited. The Pgp ATPase activity was, however, inhibited by about 20% when the nucleotide in the preincubation mixture was 2′-deoxy ATP. No inhibition was observed when the nucleotide in the preincubation mixtures was 2′-deoxy GTP, 2′-deoxy TTP, or 2′-deoxy CTP. Similarly, nucleotide diphosphates, ADP, GDP, or UDP, were unable to induce the vanadate-mediated stable Pgp ATPase inhibition under these conditions. Although I have tested all the nucleotides described in Table 1, only ATP and 2′-deoxy ATP have served as substrates of hydrolysis for Pgp in the presence of verapamil (data not shown). However, the amount of P_i released from 2′-deoxy ATP was nearly 5 times lower than the amount of P_i released from ATP under identical conditions. These observations together suggest that only those nucleotides that serve as substrates of hydrolysis participate in the vanadate-induced stable inhibition of Pgp ATPase.

To obtain the affinity constant for ATP, the vanadate-induced Pgp ATPase inhibition was performed with increasing concentrations of MgATP and the results are shown in Figure 2. The concentration of MgATP at which 50% of the Pgp ATPase inhibition (k_i) occurred was approximately 0.25 mM. This value was slightly lower than the previously determined apparent K_m value for MgATP which was approximately 0.5 mM (*18*). This difference between the apparent K_i and K_m is small, and suggests that the affinity

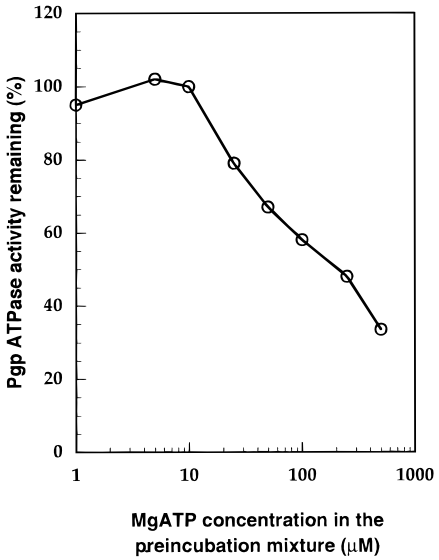

FIGURE 2: Vanadate-induced Pgp ATPase inhibition is MgATP concentration-dependent. Membranes containing wild-type Pgp (○) were incubated in a 50 mM Tris buffer, pH 7.0, containing 0.1 mM sodium orthovanadate, 50 μM verapamil, and increasing concentrations of MgATP for 5 min, pelleted by centrifugation, and washed, and the verapamil-stimulated Pgp ATPase activity in the resulting membranes was measured, as described under Materials and Methods.

of MgATP is an independent entity and is unaffected by inhibition or activation of the Pgp ATPase function. Because ATP binding is concentration-dependent with kinetic constants similar in both vanadate-induced inhibition and drug-stimulated ATP hydrolysis reactions (*18*), it is likely that ATP binds to a similar site in the Pgp molecule in both of these reactions.

(iii) Drug/Compound Requirement in the Vanadate-Induced Pgp ATPase Inhibition. I have recently demonstrated that substitution of glycine 185 in the Pgp polypeptide to valine results in increased affinities for verapamil, colchicine, and cyclosporin A. However, the affinity of vinblastine was unaffected by this substitution (*18*). To determine how this mutation influences the interactions of different drugs/compounds in the vanadate-induced stable inhibition, the wild-type and the mutant Val^{185} Pgp's were expressed in Sf9 insect cells and used in these experiments. Membranes containing the wild-type and Val^{185} Pgp's were incubated for 5 min in a 50 mM Tris Cl buffer, pH 7.0, containing 10 mM MgATP, 0.1 mM vanadate, and a wide variety of drugs/compounds. The membranes were washed, and the Verapamil-stimulated ATPase activities in the resulting membranes were then measured as described under Materials and Methods. The results are shown in Table 2. It should be pointed out that treatment of membranes with MgATP and drugs/compounds but in the absence of vanadate did not affect the Pgp ATPase activity, suggesting that high concentrations of drugs/compounds used in this study per se do not inhibit the enzyme activity. Verapamil, vinblastine, and rhodamine 123, the well-known stimulators of Pgp ATPase activity, inhibited both forms of Pgp by approximately 85%, 70%, and 60%, respectively. Interestingly, while the wild-type Pgp was inhibited by approximately 20% in the presence of colchicine and doxorubicin, the Val^{185} Pgp ATPase was inhibited by approximately 60% in the presence of both of these compounds. Interestingly, cyclosporin A

Table 2: Effect of Different Drugs/Compounds on the Vanadate-Induced Pgp ATPase Inhibition[a]

	activity inhibition (%)	
drugs/compounds	wild-type Pgp ATPase	Val^{185} Pgp ATPase
verapamil	84 ± 3	85 ± 3
vinblastine	70 ± 3	65 ± 5
colchicine	17 ± 4	61 ± 4
doxorubicin	23 ± 3	60 ± 4
rhodamine 123	58 ± 5	65 ± 4
methotrextate	7 ± 3	7 ± 3
5-fluorouracil	7 ± 3	0
ouabain	13 ± 4	16 ± 5
cyclosporin A	86 ± 4	78 ± 7

[a] In a typical preincubation reaction mixture, the Pgp-containing membranes were incubated in a 50 mM Tris Cl buffer, pH 7.0, containing 10 mM ATP, 10 mM $MgSO_4$, 0.1 mM vanadate, and 50 μM verapamil. In determining the selectivity of drugs/compound, verapamil in the preincubation mixture was replaced with an equimolar concentration of indicated compounds, except cyclosporin A, which was 5 μM. The residual verapamil-stimulated ATPase activity was determined after washing membranes as described under Materials and Methods. One-hundred percent activity is equivalent to the Pgp ATPase activity in 1 mg of membrane protein which was approximately 100 nmol/min. The values are the mean ± standard deviations of 5 experimental results.

also induced stable inhibition in the presence of vanadate and MgATP. We have shown previously that cyclosporin A inhibits the drug-stimulated Pgp ATPase activity by binding to the drug-binding site of the Pgp with high affinity (*29*). Thus, the above observations suggest that drug binding to the drug-binding site is a prerequisite for the vanadate to stably inhibit the Pgp ATPase function. In support of this contention, compounds that do not bind to the drug-binding site of the Pgp, for example, 5-fluorouracil and methotrexate, did not induce any significant Pgp ATPase inhibition. Taken together, these results indicate that the compounds that interact with and bind to the drug-binding site of the Pgp induce stable inhibition of Pgp ATPase function. Vanadate-induced Pgp ATPase inhibition obtained in the presence of different drugs/compounds was variable which may reflect on the differences in affinities of these compounds to the drug-binding site of the Pgp. The following experiments illustrate the significance of drug affinities in the stable inhibition of Pgp ATPase.

Membrane fractions containing wild-type and Val^{185} Pgp's were incubated for 5 min in a 50 mM Tris Cl buffer containing 10 mM MgATP, 0.1 mM vanadate, and varied concentrations of verapamil, washed to remove the excess ligands, and the residual Pgp ATPase activities in the resulting membranes were then measured as described under Materials and Methods. The results are shown in Figure 3. It can be seen that the wild-type and Val^{185} Pgp's were inhibited in a verapamil concentration-dependent manner, with 50% inhibition at approximately 5 and 1 μM, respectively. These values are identical to the apparent K_m values of verapamil for the wild-type (5 μM) and for the Val^{185} (1 μM) Pgp ATPases (*18*). In addition, when vinblastine was used in place of verapamil, the half-maximal inhibition of both Pgp's occurred at 0.5 μM (data not shown), which was again identical to the previously reported apparent K_m value of vinblastine for both of these Pgp forms (*18*). Thus, these observations suggest that the affinity with which a compound interacts with Pgp is a constant and is unaffected by activation or inhibition of the Pgp ATPase function.

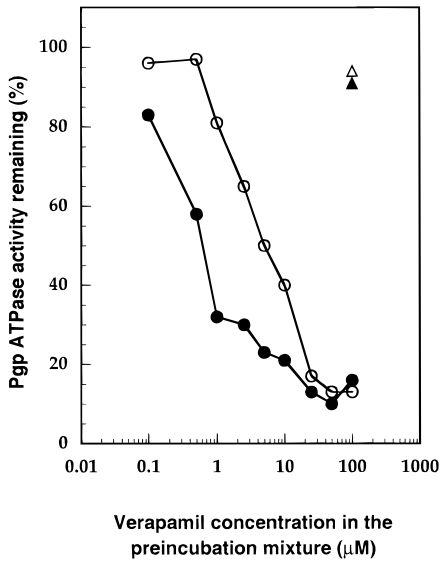

FIGURE 3: Vanadate-induced Pgp ATPase inhibition is verapamil concentration-dependent. Membranes containing the wild-type (○) and Val^{185} (●) Pgp membranes were incubated in a 50 mM Tris Cl buffer, pH 7.0, containing 10 mM MgATP, 0.1 mM sodium orthovanadate, and increasing concentrations of verapamil for 5 min, pelleted by centrifugation, and washed, and the verapamil-stimulated ATPase activity in the resulting membranes was then determined as described under Materials and Methods. For reasons of clarity, only the ATPase activities of the wild-type (△) and Val^{185} (▲) Pgp's pretreated with 100 μM vanadate and 100 μM verapamil were shown as controls.

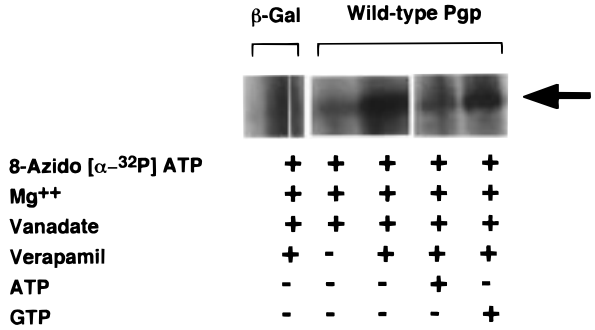

FIGURE 4: Photoaffinity labeling of Pgp. Membranes containing wild-type Pgp were incubated in a 50 mM Tris Cl buffer, pH 7.0, containing 8-azido[α−32P] ATP in the presence of different ligands as indicated, and photolabeled as described under Materials and Methods. As a control, the *E. coli* β-galactosidase expressing Sf9 insect cell membranes (β-Gal) was preincubated under conditions in which the Pgp could be photoaffinity labeled. The arrow indicates the ~130 kDa Pgp.

Photoaffinity Labeling of Nucleotide-Binding Site. To directly demonstrate that vanadate-induced stable inhibition requires binding of Mg^{2+}, ATP, and compounds that interact with drug-binding site, the wild-type Pgp was preincubated with Mg^{2+}, 8-azido[α-^{32}P]ATP, and verapamil in different combinations and photolabeled as described under Materials and Methods. The results are shown in Figure 4. In the presence of vanadate, Mg^{2+}, and verapamil, a single ~130 kDa protein was photolabeled by 8-azido[α-^{32}P]ATP (lane 3), which was absent in the membranes prepared from Sf9 insect cells infected with β-Gal BV (lane 1). Immunostaining of this blot with a Pgp-specific antibody, C-219, suggested that this ~130 kDa protein is Pgp (data not shown). Interestingly, when verapamil was omitted from this preincubation mixture, 8-azido[α-^{32}P]ATP labeling of Pgp was

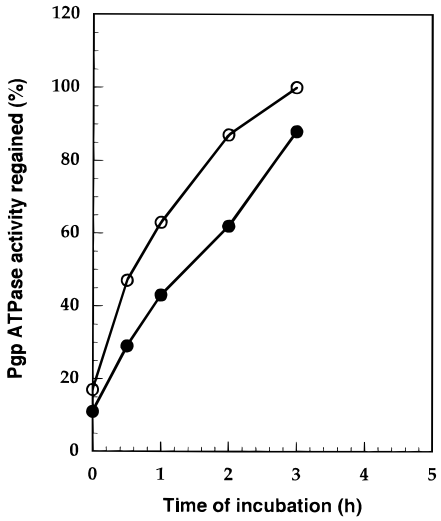

FIGURE 5: Pgp ATPase inhibition is reversible. The wild-type (○) and the Val[185] (●) Pgp ATPases were first inhibited in the presence of 10 mM MgATP, 0.1 mM sodium orthovanadate, and verapamil (50 and 10 μM in the preincubation mixtures containing wild-type and Val[185] Pgp, respectively). The membranes were washed, resuspended in a 40 mM Tris, 1 mM EGTA buffer, pH 7.0, with HCl, and incubated at 37 °C. Aliquots were taken at regular time intervals and assayed for the verapamil-stimulated ATPase activity.

significantly reduced (lane 2), suggesting that drug binding is essential for 8-azido[α-^{32}P]ATP binding and labeling. Inclusion of 1 mM ATP in the preincubation mixture also reduced the amounts of photoaffinity labeling of Pgp (lane 4) to the basal level, suggesting that 8-azido[α-^{32}P]ATP binds to the ATP-binding site. Interestingly, when 5 mM GTP was included, there was no reduction in the 8-azido[α-^{32}P]-ATP labeling of Pgp (lane 5), suggesting that GTP does not compete with the 8-azido[α-^{32}P]ATP-binding site. This observation further corroborates our results that GTP is not a hydrolyzable substrate of Pgp and does not interact with its ATP-binding site. Taken together, these direct observations clearly demonstrate that vanadate-induced stable inhibition of Pgp requires binding of Mg^{2+}, ATP, and verapamil to their respective binding sites in the Pgp.

Reactivation of the Inhibited Pgp ATPase. To determine whether the vanadate-induced Pgp ATPase inhibition is reversible and the reactivation of enzyme activity is influenced by affinities of the interacting ligands, the membranes containing wild-type and Val[185] Pgp's were incubated with MgATP, vanadate, and verapamil, washed, and then resuspended in minimal volumes of glycerol buffer. Typically, ~85% ATPase activity of both forms of Pgp is inhibited. The membrane suspension was then incubated at 37 °C, and at regular time intervals, aliquots were taken and assayed for verapamil-stimulated ATPase activity. The results are shown in Figure 5. Both the wild-type and Val[185] Pgp's regained verapamil-stimulated ATPase activity in time, with 50% reactivation at 30 and 80 min, respectively. These results suggest that vanadate-induced inhibition is reversible. Because verapamil binds to Val[185] Pgp 5 times more strongly than to the wild-type Pgp (*18*), the increase in $t_{1/2}$ of reactivation of Val[185] Pgp is likely due to the tight binding of verapamil to the Val[185] Pgp, thus leading to an increased stability of the inhibited Pgp species.

DISCUSSION

In this paper, the interactions of ligands participating in the catalytic cycle of the Pgp are characterized to search for a relationship between the events of ATP hydrolysis and drug transport. Vanadate, a well-known P_i analogue, has been used extensively to characterize the intermediate steps of phosphotransfer reactions mediated by several enzymes (*32*). In the case of myosin, vanadate has been shown to bind to the active site in the presence of MgADP and to form an inactive ternary complex, myosin·MgADP·vanadate. The X-ray diffraction (*30*) and spin-label experiments (*31*) have suggested that this complex is conformationally analogous to the transition-state myosin·MgADP·P_i species, which is believed to be a key intermediate in the energy transduction process. Thus, vanadate-induced trapping of ligands at the active site will provide ways of identifying these sites by protein chemical methods and allow further understanding of enzyme catalysis. Senior and his colleagues have first applied this methodology to the Chinese hamster Pgp (*23*). These studies have indicated that the vanadate-induced inhibition of Chinese hamster Pgp occurs in the presence of a variety of nucleotides including ATP, ADP, GTP, ITP, UTP, and CTP, and the inhibition does not appear to depend on the presence of drugs/compounds. The results presented in this paper strongly suggest that the human Pgp exhibits high nucleotide specificity in the vanadate-induced ATPase inhibition and that this inhibition strictly depends on the presence of drugs/compounds. Vanadate, in the presence of ligands participating in the ATP hydrolysis together with drugs/compounds known to interact with the drug-binding site of the Pgp, transforms the catalytically active human Pgp into an inactive species. Thus, a strict dependence of ligands participating in the ATP hydrolysis and drug-transport reactions in the vanadate-induced inhibition suggests that these two functions of the Pgp are tightly coupled.

One of the interesting and potentially important features reported in this paper is the apparent influence of nucleotides on the vanadate-induced inhibition of Pgp. Among the several nucleotides tested, only ATP or 2′-deoxy ATP is able to participate in the vanadate-induced inhibition of the Pgp ATPase (Table 1). The fact that Pgp was photoaffinity labeled by 8-azido[α-^{32}P]ATP in the presence of Mg^{2+}, verapamil, and vanadate, and that this labeling was reduced in the presence of ATP, firmly establishes that ATP is trapped in the stably inhibited Pgp species. Although it is surprising that GTP, β,γ-methylene ATP, and ADP were unable to participate in the vanadate-induced inhibition, the answer appears to be in their inability to serve as substrates of hydrolysis. It has been observed that among all the nucleotides described in Table 1, only ATP and 2′-deoxy ATP were hydrolyzed by the human Pgp in the presence of verapamil. Further analyses of the interactions of GTP, β,γ-methylene ATP, and ADP with the ATP-binding site in the Pgp molecule have suggested that although GTP does not interact in any way with the ATP-binding site, β,γ-methylene ATP, and ADP clearly do, with affinities of 1.25 and 0.3 mM, respectively (data not shown). These observations suggest that β,γ-methylene ATP and ADP bind to the ATP-binding site, whereas GTP does not. Although studies from the laboratory of Senior (*23*) have suggested that the trapped nucleotide is ADP, results presented in this paper provide

further insights into the pathway leading to the formation of ADP at the active site. For instance, the fact that the inhibition is dependent on the hydrolyzable nucleotides points out that the bound nucleotide undergoes hydrolysis to form ADP at the active site. The fact that ADP and ADP + P_i failed to participate in the Pgp ATPase inhibition is consistent with the view that the formation of Pgp·MgATP·verapamil·vanadate complex is a prerequisite for the Pgp·MgADP·verapamil·vanadate complex formation.

Another striking feature of the results presented in this paper is the importance of drug/compound binding in the vanadate-induced stable inhibition of Pgp ATPase. Vanadate-induced inhibition is strictly dependent on drugs/compounds that interact with the drug-binding site of the Pgp. A wide range of compounds is effective (Table 2), and the variable extent of Pgp ATPase inhibition obtained in their presence indicates a substantial specificity in their interactions with the drug-binding site. In a previous communication, I have compared the biochemical properties of the wild-type and Val^{185} Pgp's, which suggested that there are two drug-binding sites in the Pgp molecule and that mutation at amino acid 185 in the polypeptide alters the affinities of the interacting drugs/compounds (*18*). Similarly, the use of wild-type and Val 185 Pgp's in the present study also provided important clues as to the biochemical properties of the vanadate-induced stably inhibited Pgp species. The demonstration that verapamil interacts with Val^{185} Pgp 5 times more strongly than with the wild-type in the vanadate-induced inhibition (Figure 3) is consistent with the previous observations that verapamil also interacts with Val^{185} Pgp 5-fold more strongly than with the wild-type Pgp in the ATPase measurements (*18*). These observations together extend strong support to the notion that the verapamil-binding site is identical in both of these reactions. The studies on the interactions of methotrexate and 5-fluorouracil, compounds that do not interact with the Pgp drug-binding site, suggest that drug-binding site occupancy is a prerequisite condition for a stable inhibition. Interestingly, Pgp ATPase function was strongly and stably inhibited by vanadate in the presence of cyclosporin A. Although cyclosporin A interacts with the drug-binding site with high affinity, it fails to stimulate the Pgp ATPase function (*29*). In such an event, how can one explain hydrolysis of the bound ATP and stability of the inhibited Pgp species, by the rule stipulated above to explain the vanadate-induced stable inhibition observed in the presence of verapamil? Although our previous observations did not indicate ATP hydrolysis in the presence of cyclosporin A (*29*), it is reasonable to consider that Pgp undergoes a partial turnover without the release of bound cyclosporin A or the nascently formed ADP from their binding sites. Such interpretation seems to provide an explanation for the observation of vanadate-induced stable inhibition of Pgp ATPase in the presence of cyclosporin A.

The differences in the reactivation kinetics of the inhibited Pgp appear to provide important clues as to the factors influencing its stability. Results shown in Figure 5 that the $t_{1/2}$ of reactivation of the Val^{185} Pgp is nearly 3 times longer than that of the wild-type Pgp ATPase suggest that the amino acid 185 in the Pgp polypeptide may have an indirect role in the stabilization of the inhibited Pgp. It is worth noting that although the affinities with which vanadate and MgATP interact with the wild-type and the Val^{185} Pgp's are nearly

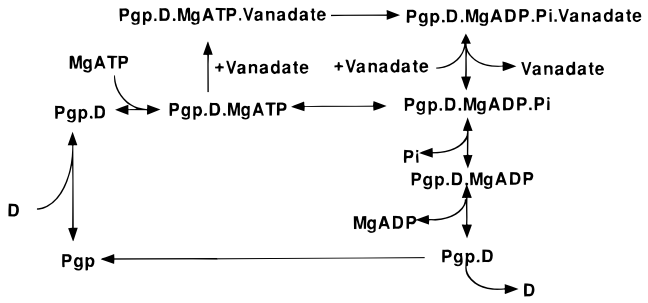

FIGURE 6: A schematic representation of Pgp ATPase catalytic cycle: D, drug/compound.

identical, the affinity of verapamil was different and 5-times higher with the Val^{185} Pgp (*18*). Thus, the increased stability of the Val^{185} Pgp can be directly attributed to the affinity of verapamil binding. Taken together, the stability of the inhibited Pgp species is dependent on overall affinities of the individual ligands participating in its formation.

Finally the results presented in this paper are of potential interest and are pertinent to the catalytic mechanism of the Pgp. The experimental findings that ATP and drug binding are prerequisites for the Pgp ATPase inhibition have raised a distinct possibility that the entire Pgp-mediated active drug transport may be no more complicated than the mechanism of enzyme catalysis that employs principles of the transition-state theory. Although the transition state of an enzyme formed by the catalytic cycle reactants is unfortunately too short-lived, it would be desirable to study structures approaching this state by the use of their structural analogues. Because vanadate is a phosphate analogue, the structure of the stably inhibited Pgp species composed of MgATP, drug, and vanadate may represent a transition-state configuration of the Pgp catalytic cycle. Although the results do not exactly suggest a catalytic pathway, a simple scheme with different possible ligand-bound Pgp intermediates is shown in Figure 6. The reaction pathway is intentionally lacking several details about the conformational states of Pgp, ATP hydrolysis, and drug movement. The fact that Pgp does not have high-affinity ATP binding in the absence of drug (Figure 4) suggests that the ATP-binding site(s) is occluded in the absence of drug binding. This strengthens our observations that Pgp does not exhibit ATPase activity in the absence of a transport substrate. Upon binding of MgATP and drug/compound substrate, the reaction pathway probably proceeds through an intermediate depicted as the Pgp·MgADP·P_i·drug complex, followed by sequential release of P_i, MgADP, and drug. Although the precise location of vanadate binding is unclear at present, the following considerations are noteworthy in this context. First, because vanadate-induced inhibition requires ATP or a hydrolyzable nucleotide triphosphate, vanadate likely binds to the MgATP- and verapamil-bound Pgp and forms Pgp·MgATP·verapamil·vanadate complex. Hydrolysis of bound ATP will transform this complex into Pgp·MgADP·P_i·verapamil·vanadate complex. It is possible that vanadate binds to a Pgp·MgADP·P_i·verapamil configuration assumed after hydrolysis of the bound ATP. Because there are two putative ATP-binding sites in the Pgp molecule, it can be assumed that vanadate binds to the γ-phosphate-binding region of the second ATP-binding site. Alternatively, vanadate binds to the γ-phosphate-binding region of the bound ATP only after hydrolysis and

subsequent removal of the terminal phosphate. Further analysis of the inhibited species will provide insights into the energy-dependent drug transport by this transporter.

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