CORRELATION BETWEEN STEADY-STATE ATP HYDROLYSIS AND VANADATE-INDUCED ADP TRAPPING IN HUMAN P-GLYCOPROTEIN: EVIDENCE FOR ADP RELEASE AS THE RATE LIMITING STEP IN THE CATALYTIC CYCLE AND ITS MODULATION BY SUBSTRATES.

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Running Title: Effect of Substrates on the Catalytic Cycle of P-glycoprotein

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Abbreviations: ABC, ATP-binding cassette; MDR, multidrug resistance; DMSO, dimethyl sulfoxide; NADH, nicotinamide adenine dinucleotide–reduced form; OG, octyl β-D-glucopyranoside; OD, optical density; PEP, phosphoenolpyruvate; Pgp, P-glycoprotein; P1, inorganic phosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPP⁺, tetraphenylphosphonium chloride; Vi, vanadate.
SUMMARY

P-glycoprotein (Pgp) is a transmembrane protein conferring multidrug resistance to cells by extruding a variety of amphipathic cytotoxic agents using energy from ATP hydrolysis. The objective of this study was to understand how substrates affect the catalytic cycle of ATP hydrolysis by Pgp. The ATPase activity of purified and reconstituted recombinant human Pgp is measured using a continuous cycling assay. Pgp hydrolyzes ATP in the absence of drug at a basal rate of 0.5 $\mu$mol$\cdot$min$\cdot$mg$^{-1}$ with a $K_m$ for ATP of 0.33 mM. This basal rate can be either increased or decreased depending on the Pgp substrate used, without an effect on the $K_m$ for ATP or 8-azidoATP and $K_i$ for ADP suggesting that substrates do not affect nucleotide binding to Pgp. Although, inhibitors of Pgp activity, cyclosporin A, its analogue PSC833, and rapamycin decrease the rate of ATP hydrolysis with respect to the basal rate, they do not completely inhibit the activity. Therefore, these drugs can be classified as substrates. Vanadate (Vi)-induced trapping of [$\alpha$-$^{32}$P]-8azidoADP was used to probe the effect of substrates on the transition-state of the ATP hydrolysis reaction. The $K_m$ for [$\alpha$-$^{32}$P]-8azidoATP (20 $\mu$M) is decreased in the presence of Vi; however, it is not changed by drugs such as verapamil or cyclosporin A. Strikingly, the extent of Vi-induced [$\alpha$-$^{32}$P]-8azidoADP trapping correlates directly with the fold-stimulation of ATPase activity at steady state. Furthermore, $P_i$ exhibits very low affinity for Pgp ($K_i$ ~30 mM for Vi-induced 8-azidoADP trapping). In aggregate, these data demonstrate that the release of Vi trapped [$\alpha$-$^{32}$P]-8azidoADP from Pgp is the rate-limiting step in the steady-state reaction. We suggest that substrates modulate the rate of ATPase activity of Pgp by controlling the rate of dissociation of ADP following ATP hydrolysis and that ADP release is the rate-limiting step in the normal catalytic cycle of Pgp.
INTRODUCTION

Multidrug resistance, the reduced sensitivity to a variety of structurally unrelated, hydrophobic, chemotherapeutic agents, is a major problem in cancer treatment. This phenomenon is often associated with the overexpression of the human multidrug resistance gene (*MDR1*) (1,2). *MDR1* encodes a 170-kDa plasma membrane protein, P-glycoprotein (Pgp), that uses the energy from ATP hydrolysis to expel a variety of anticancer drugs from cells, thus making them ineffective during chemotherapy. The secondary structure of Pgp is predicted to consist of two homologous halves each containing six putative transmembrane helices and a nucleotide-binding domain. These structural elements are common to a large family of membrane transporters called the ATP-binding cassette (ABC) superfamily (3,4).

The widely accepted hypothesis of Pgp function is that substrate transport is coupled to ATP hydrolysis. However, the mechanism for this reaction is not well understood. In the absence of added substrate, Pgp catalyzes basal ATP hydrolysis (ATPase activity). This basal activity has been suggested to occur due to an endogenous lipid substrate(s) or an uncoupling of ATP hydrolysis and drug extrusion (5,6). The basal rate is stimulated by adding any one of a variety of hydrophobic drug substrates; these drugs bind with unique apparent affinities and affect the ATPase activity of Pgp to varying degrees (7-9). However, a detailed assessment of the kinetic parameters of ATP hydrolysis in the presence of amphipathic drugs for the identification of the rate-limiting step in the catalytic cycle has not been carried out.
Expression, purification, and reconstitution procedures of endogenous and six-histidine-tagged (His6) human Pgp in a heterologous expression system are well-described (10-12). Studies on the ATPase activity of Pgp in crude membrane preparations have indicated various drug-stimulated effects on the ATPase activity of Pgp (5); such experiments with crude protein can be difficult to interpret. Clearly, when purifying Pgp, the lipid environment of this membrane protein affects the ATPase activity and should remain constant for comparison of drug-stimulated activities (13-16).

Kinetic schemes for the catalytic cycle of Pgp has been proposed based on binding studies and Vi-induced ADP trapping experiments (17-21). Vi is a proposed transition-state analog that replaces P₁ immediately after its release upon ATP hydrolysis. No phospho-enzyme intermediate of Pgp has been identified in the catalytic cycle of Pgp indicating that all intermediates in the Pgp reaction are non-covalent (18). Senior and his colleagues have extensively characterized the Vi-induced ADP trapping reaction (18,19) and implied that P₁ release precedes ADP release and ADP release is the most likely rate-limiting step in catalysis (22). Our objective in this study is to understand how Pgp drug substrates might affect the overall kinetic mechanism of Pgp. Kinetic constants for various drugs that affect the steady-state ATPase activity of Pgp are quantified, and the extent of Vi-induced ADP trapping are compared in the presence and absence of various Pgp drug substrates. The correlation of these values has mechanistic implications. Our results suggest that ADP release is a rate-limiting step in the catalytic cycle of Pgp, and substrates and modulators exert their effect on ATPase activity by modulating this step.
EXPERIMENTAL PROCEDURES

Materials. Octyl β-D-glucopyranoside (OG) and cyclosporin A were purchased from Calbiochem (San Diego, CA). ATP (disodium salt), ADP, progesterone, prazosin, valinomycin, verapamil, vinblastine, and staurosporin were purchased from Sigma (St. Louis, MO); tetraphenylphosphonium chloride (TPP⁺) from Aldrich (Milwaukee, WI); tamoxifen from Research Biochemicals International (Natick, MA); rapamycin was obtained from the NCI Developmental Therapeutics Program (Bethesda, MD); and PSC833 was the generous gift of (Novartis Corp., East Hanover, NJ). Acetone-ether washed E.coli bulk phospholipids, egg phosphatidylcholine, phosphatidylserine and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). Cycling components, as described below, were purchased from Boehringer Mannheim (Indianapolis, IN). 8-azidoATP and [α⁻³²P]-8azidoATP (10-20 Ci/mmol) was purchased from ICN Biomedicals (Irvine, CA).

Expression and Purification of Pgp. Human Pgp was expressed and purified as previously described (11) with minor changes. Briefly, (His)₆-tagged Pgp was expressed using Trichoplusia ni (High Five™, Invitrogen, San Diego, CA) insect cells grown in monolayer cultures and infected with a recombinant baculovirus BV- MDRI (H₆) which contains a human MDR1 that encodes Pgp. Crude Pgp-containing membranes were prepared by Dounce homogenization under hypotonic conditions (23), and membrane proteins were solubilized with OG (1.25%) in the presence of 20% glycerol and lipid-mixture (0.1%) (see Routine Procedures). Solubilized proteins were subjected to metal affinity chromatography (Talon resin from Clontech, Palo Alto, CA) in the presence of 0.95% OG and 0.04% lipid; 80% purified Pgp was
eluted with 100 mM imidazole (11). Pgp in 100 mM imidazole fraction was then concentrated (Centriprep-50, Amicon, Beverly, MA) to approximately 0.5 mg/ml and stored at –70°C. Pgp was identified by immunoblot analysis (11) and quantitated by Amido Black protein estimation method as previously described (24).

Reconstitution of Pgp. Purified Pgp was combined with a lipid mixture (acetone-ether washed E.coli bulk phospholipid, phosphatidylcholine, phosphatidylserine, and cholesterol (60:17.5:10:12.5 w/w) (8)), previously sonicated for 20 min in the ratio of 1:8 (protein: lipid, by weight). This reconstitution mixture was dialyzed extensively (3 hrs with 4 buffer changes at 4°C) with dialysis buffer (50 mM MES-Tris, pH 6.8, 1 mM EGTA, 1 mM DTT, and 0.1% aprotinin) using Slide-A-Lyzer cassettes (10K cutoff from Pierce, Rockford, IL). Pgp-containing proteoliposomes were collected by centrifugation at 140,000 X g for 1 hr at 4°C and resuspended in dialysis buffer. Protein concentration was estimated by the Amido Black assay as described above.

ATPase Assays. ATPase activity of purified, reconstituted Pgp was measured by two methods; the endpoint, inorganic phosphate (P_i) assay and the continuous cycling assay. In both assays, Pgp-specific activity was recorded as the V_i (0.3 mM) -sensitive ATPase activity. Various test agents were added from 100x stock solutions in DMSO so that the DMSO concentration was no greater than 1%; this concentration of DMSO had no effect on the activity of Pgp or the cycling assay components. For the P_i assay, the amount of inorganic phosphate released over 20 minutes at 37°C was measured. ATPase assay buffer (50 mM MES-Tris, pH 6.8, 50 mM N-methyl-D-glucamine chloride, 5 mM NaN_3, 1 mM EGTA, 1 mM ouabain, and 2
mM DTT) was combined with either 5 mM MgCl₂ or CoCl₂, 0.5 – 2 µg purified, reconstituted Pgp, and various Pgp drug substrates for a 5 min preincubation at 37°C. The reaction was initiated by the addition of 5-7.5 mM ATP and quenched with SDS (2.5% final concentration); the amount of P₁ released was quantitated using a colorimetric method as previously described (11). For the cycling assay, cycling components (3 mM PEP, 0.33 mM NADH, and 10 units/ml both pyruvate kinase and lactate dehydrogenase) were added to the ATPase assay buffer (described above) with 10-15 mM MgCl₂ to link the hydrolysis of ATP directly with the oxidation of NADH (12). Purified, reconstituted Pgp (1 – 10 µg in 100 µl assay volume) was preincubated with various Pgp drug substrates at 37°C in a temperature-controlled, 96-well plate spectrophotometer (Spectra MAX 250, Molecular Devices, Sunnyvale, CA). The reaction was initiated by the addition of ATP and monitored at OD₃₄₀nm using SoftMax Pro 2.4 software (Molecular Devices, Sunnyvale, CA) for 5–10 minutes. The rate of change in absorbance was converted to nmol NADH oxidized per minute using an NADH standard curve; this value is equivalent to nmol ATP hydrolyzed per minute.

Kinetic Analysis. The drug-stimulated ATPase activities in the presence of saturating ATP concentrations (5-7.5 mM) and various Pgp drug substrates were fit to Equation 1:

\[
\frac{\nu}{E} = \frac{V \cdot S}{K_{app} + S} + V_b
\]

(1)

Where \(\frac{\nu}{E}\) is the ATPase activity at given concentrations of substrates, \(V\) is \(V_{max} - V_b\) with \(V_{max}\) as the maximal activity, \(S\) is the drug substrate concentration, \(K_{app}\) is the apparent concentration at half-maximal activity, and \(V_b\) is the basal rate in the absence of added drug. The fold-stimulation of ATPase activity by a given substrate is calculated by \(V_{max}/V_b\). Michaelis-Menten parameters were determined for ATP and 8-azidoATP in the presence of saturating Pgp drug.
substrates, verapamil (50 µM) and cyclosporin A (10 µM); for basal activity (in the absence of added drug) an equivalent volume of DMSO was added. ATPase activities using various nucleotide concentrations were fit to Equation 2:

$$\frac{\nu}{E} = \frac{V_{\text{max}} \cdot [\text{NTP}]}{(K_m + [\text{NTP}])}$$  \hspace{1cm} (2)

Where NTP is the concentration of ATP or 8-azido-ATP, and $K_m$ is the concentration of NTP at half-maximal activity. Inhibition constants for ADP were determined using various concentrations of ADP and ATP in the presence and absence of 50 µM verapamil. ATPase activities were fit to Equation 3 for competitive inhibition:

$$\frac{\nu}{E} = \frac{V_{\text{max}} \cdot [\text{ATP}]}{(K_m (1 + [\text{ADP}]/K_i)+ [\text{ATP}])}$$  \hspace{1cm} (3)

Where $K_i$ is the concentration of ADP at half-maximal inhibition. All curve fits in kinetic analyses were performed using Prism 2.0 software for Power-Mac (GraphPad, San Diego, CA).

**Vanadate-induced 8-azidoADP Trapping in Pgp.** Vi-induced 8-azidoADP trapping assays contained ATPase assay buffer, 0.3 mM Vi, either MgCl$_2$ or CoCl$_2$ (5 mM), 0.2 – 0.8 mg/ml purified Pgp (reconstituted), Pgp drug substrate, and 5 – 100 µM [α-32P]-8azidoATP (2.5-10 µCi/nmole). Reactions were preincubated in low light or semi-darkness in the absence of [α-32P]-8 azidoATP at 37°C for 5 min, initiated by the addition of [α-32P]8-azidoATP, and quenched by the addition of ice cold ATP (12.5 mM). Reactions were exposed to UV light (UV-A long wave-F15T8BLB tubes, 365 nm wavelength, PGC Scientifics, Gaithersburg, MD) on ice for 10 min and subjected to SDS-PAGE and autoradiography. The extent of [32P] labeling was quantified using the Storm 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA). The quantification of 32P incorporation/mg Pgp was performed using SDS-PAGE samples, previously
dried for phosphorimager detection, then dissolved in Solvable (Packard, Meriden, CT) before scintillation counting as described previously (25).

**Determination of the** $K_m$ **for 8-azidoATP during Vi-induced Trapping.** Purified P-gp was reconstituted into proteoliposomes and incubated in the ATPase assay buffer containing 0.3 mM Vi and increasing concentrations (1-75 µM) of $[^{32}P] - 8$azidoATP (3-5 µCi/nmole) in the dark at 37°C for 3 min. The reaction was stopped by addition of 12.5 mM ice-cold ATP and placing the sample on ice. Trapping of Pgp into the Pgp•Mg8AzidoADP•Vi conformation was carried out under basal conditions and in the presence of either 50 µM verapamil or 10 µM cyclosporin A. Following SDS PAGE, on an 8% Tris-glycine gel, the radioactivity in the Pgp bands was quantified on a STORM 860 phosphorimager system. The $K_m$ values for 8azidoATP under basal conditions and in the presence of saturating concentrations of verapamil and cyclosporin A were obtained by fitting the data to Equation 2 using the software Prism as described above.

**Inhibition of Vi-induced 8-azidoADP Trapping by** $P_i$. Proteoliposomes containing purified Pgp (5-10 µg) were incubated in the ATPase assay buffer containing 0.30 mM Vi. The proteoliposomes were then treated with DMSO (control), 50 µM verapamil or 10 µM cyclosporin A either in the presence of 0 to 150 mM KH$_2$PO$_4$ (pH 6.8) or KCl. Finally, 50 µM $[^{32}P] - 8$azidoATP (2.5-5 µCi/nmole) was added in the dark and incubated at 37°C for 5 min. The reaction was stopped by quenching with 12.5 mM ice-cold ATP solution and placing on ice. Following SDS PAGE, on an 8% Tris-glycine gel, the extent of trapping of 8-azidoADP was determined as described above.
**Binding of \( \alpha^{32}P \)-8-azidoATP to Pgp.** Proteoliposomes (5-10 µg protein) were incubated in the ATPase assay buffer in the presence of DMSO, 50 µM verapamil, or 10 µM cyclosporin A for 5 min at 37°C and transferred to ice. After 5 min on ice, 10 µM \( \alpha^{32}P \)-8azidoATP (5-10 µCi/nmole) was added to each sample in the dark and incubated at 4°C for 5 min. The samples were then irradiated with UV light (365 nm) on ice (4°C) as described above. Ice cold ATP (12.5 mM) was added to displace excess non-covalently bound \( \alpha^{32}P \)-8azidoATP. Excess nucleotides and Vi were removed by centrifugation at 300,000 X g at 4°C for 10 min by using S120-AT2 rotor in a RC-M120EX micro-ultracentrifuge (Sorvall, Newtown, CT) and the pellet resuspended in 1X SDS PAGE sample buffer. Following SDS PAGE on a 8% Tris-glycine gel at constant voltage, gels were dried and exposed to Bio-Max MR film (Eastman Kodak, Rochester, NY) at -70°C for 12-24 h. The radioactivity incorporated into the Pgp band was quantified using the STORM 860 phosphorimager and the software ImageQuaNT.

**Routine Procedures.** The lipid mixture was made by combining acetone/ether washed *E. coli* bulk phospholipids, phosphatidylcholine, phosphatidylserine, and cholesterol (Avanti Polar Lipids, Alabaster, AL) in the ratio of 60:17.5:10:12.5 by weight and evaporating to dryness under N₂ gas (26). This dried stock was stored at –70°C until resuspension at 50 mg/ml in a 2 mM β-mercaptoethanol solution. SDS-PAGE was performed using pre-cast 8% Tris-Glycine gels (Novex, San Diego, CA). Immunoblot analyses were performed as previously described (27) using the monoclonal antibody, C219 (a gift from Centocor). The Colloidal Blue Staining Kit (Novex, San Diego, CA) was used for total protein staining of SDS-PAGE samples. Sodium orthovanadate (Sigma, St. Louis, MO) was prepared by boiling a 50 mM solution in water for 3 min and concentration was determined by using molar absorbance \((\lambda_{268nm} = 3600 \text{ M}^{-1})\).
RESULTS

Production of Pure, Catalytically Active Human Pgp. The previously described method was adopted for the purification of large amounts of pure Pgp (11). From approximately $4 \times 10^9$ cells of baculovirus-infected High Five insect cells in suspension culture, 500-600 mg of crude membrane protein was routinely obtained. 60-70% of this crude membrane protein was recovered after solubilization with octylglucoside, and 4-6 mg of purified Pgp was collected following metal affinity chromatography. The reconstitution of purified Pgp into an artificial lipid bilayer, which is required to obtain a Pgp preparation totally free of the detergent, typically yielded 65% recovery, a value common among dialysis reconstitution procedures for Pgp (12, 26, 28).

Measuring the ATPase Activity of Pgp. The ATPase activity of purified, reconstituted Pgp was routinely measured using two techniques: 1) an endpoint, $P_i$ release assay measuring the amount of $P_i$ released by the hydrolysis of ATP in a given amount of time and 2) a continuous cycling assay linking the hydrolysis of ATP to the oxidation of NADH using pyruvate kinase, lactate dehydrogenase, and their substrates. The cycling assay was preferred to the end point $P_i$ assay because of its ability to monitor the ATPase reaction continuously in real time; in this way, the rates of ATPase activity were easily identified as steady-state, linear rates. While the cycling assay was used to quantitate the kinetics of ATP and all Pgp drug substrates, some drawbacks were inherent in the method. As the cycling assay measures decrease in the absorbance at 340 nm resulting from the oxidation of NADH, activity measurements using 8-azidoATP, a light-sensitive substrate, were impossible and, therefore, quantitated using the $P_i$ assay. Furthermore,
the ATPase activity could not be monitored in the presence of ADP due to its stimulatory effect of cycling in the absence of ATP hydrolysis.

A typical ATPase cycling experiment is shown in Figure 1, where the rate (mOD$_{340}$/sec) of ATPase activity specific to Pgp is determined as the Vi-sensitive activity in the presence or absence of verapamil. The rate of change of absorbance per second is converted to nmol NADH oxidized•min$^{-1}$ using an NADH standard curve. This value is directly comparable to the ATPase activity of Pgp recorded as nmol ATP hydrolyzed•min$^{-1}$•mg$^{-1}$. Typically, verapamil-stimulated ATPase activity of purified and reconstituted Pgp was 0.6 – 1.2 µmol ATP hydrolyzed•min$^{-1}$•mg$^{-1}$, which is consistent with the values obtained using the P$_i$ assay. This specific activity is slightly lower than previously reported for the human Pgp reconstituted by using the rapid dilution procedure (11).

**Steady-State Kinetics with Various Pgp Drug Substrates.** To characterize how human Pgp uses different drugs to stimulate ATPase activity in a concentration-dependent manner, several Pgp substrates were assayed over large concentration ranges. A true $K_m$ value cannot be obtained due to the ATPase activity in the absence of added drug substrates (the basal rate, fold-stimulation = 1.0). A similar parameter, $K_{app}$, is used to describe the concentration of progesterone, prazosin, TPP$^+$ ion, valinomycin, verapamil, cyclosporin A, PSC833 (a cyclosporin A analog), and rapamycin at half-maximal ATPase activity (see Table 1). These drugs either increased (fold-stimulation > 1.0) or decreased (fold-stimulation < 1.0) the ATPase activity of Pgp. The values for $K_{app}$ (often referred to as $K_m$ or $K_{0.5}$) and fold-stimulation vary greatly throughout the literature. Much of the published data using either human or Chinese
hamster Pgp is consistent with the values obtained in this study (6,11,20,28-30). However, significant differences in progesterone stimulation (30-34) and varied data using verapamil stimulation (6,7,11,28,30-32,34-39) should be noted. In this study, many values for $K_{\text{app}}$ are given as upper or lower limits due to concentration limitations. For these experiments, the drug concentrations were at least in 5-fold excess of the amount of Pgp (nmol: nmol) and were considered to be saturated if a 10-fold increase in drug concentration did not affect the ATPase activity.

Figure 2A demonstrates both increased and decreased ATPase activity in the presence of selected agents. In the presence of saturating Mg•ATP and increasing concentrations of either prazosin or verapamil, the ATPase activity increases to a saturable $V_{\text{max}}$ value. The value of $K_{\text{app}}$ for verapamil (4.7 µM) is less than for prazosin (16 µM) indicating a higher affinity for verapamil while the fold-stimulation is greater for prazosin (4.5-fold) with respect to verapamil (2.0-fold); similar values have been previously reported (6,11,28-32,34-36,39). Interestingly, drugs typically considered Pgp inhibitors, like cyclosporin A, its analog PSC833, and rapamycin, are clearly depicted as substrates (see Table 1 and data with cyclosporin A is given in Figure 2A insert). Upon saturation, these drugs support ATPase activity by Pgp; however, the rate is less than the basal rate in contrast to a previous report (11). Unfortunately, values of $K_{\text{app}}$ for these drugs are not able to be quantitated due to the high concentrations (0.4 µM) of Pgp required to assay for activity in either the cycling or Pi assay. However, data describing cyclosporin A as an inhibitor report low concentrations (74–400 nM) for maximal effect (6,33,40,41) so the saturation of these compounds is likely.
Effect of Selected Drug Substrates on the Affinity of Pgp for Nucleotides. Pgp substrates, like verapamil and cyclosporin A, affect the rate at which Pgp hydrolyzes ATP; this effect is often described as fold-stimulation with respect to the basal rate of ATPase activity. However, these drugs do not change the ability of Pgp to utilize nucleotides. Figure 2B depicts the differences in maximal ATPase activity in the absence (basal) and in the presence of verapamil or cyclosporin A, and suggests the similarities in values of $K_m$, the concentration required for half-maximal stimulation. Table 2 reports the values of $V_{max}$ and $K_m$ for ATP (0.22, 0.33, and 0.26 mM) and 8-azidoATP (0.4, 0.6, and 0.5 mM) in the presence of verapamil, no drug, and cyclosporin A, respectively. The $K_m$ values for ATP are similar to those reported previously by us (8,11). Clearly, the values of $K_m$ for either nucleotide are unchanged in the absence (basal) or presence of verapamil or cyclosporin A while the values of $V_{max}$ remain constant in relation to each other (fold-stimulation for verapamil: basal: cyclosporin A = 2:1:0.5). Additionally, the values of $K_i$ for ADP are similar in the presence ($K_i = 0.5$ mM) and absence of verapamil ($K_i = 0.3$ mM); these data display competitive inhibition patterns with respect to ATP by intersecting on the y-axis of a Lineweaver-Burke plot (data not shown) indicating that ATP and ADP bind to the same site. Other workers also have reported similar inhibition by ADP (11,28,39,42).

Vanadate-induced $[^{32}P]8$-azidoADP Trapping in the Presence of Various Pgp Drug Substrates. Under hydrolysis conditions (assay buffer, pH 6.8 at 37° C), Pgp is incubated with $[^{32}P]$-8azidoATP, MgCl$_2$ or CoCl$_2$, and Vi (0.3 mM) in the presence or absence of Pgp drug substrates. The reaction proceeds through ATP hydrolysis to products ($[^{32}P]$-8azidoADP + Pi) before Vi substitutes for Pi and traps $[^{32}P]$-8azidoADP on the enzyme (18). The reaction is quenched by adding excess ATP (12.5 mM) at 4° C to inhibit any non-specific $[^{32}P]8$-
azidoATP binding and to measure only the extent of trapped or occluded di-phosphate nucleotide. Following UV crosslinking and gel electrophoresis, the [$\alpha$-$^{32}$P]8-azidoADP incorporated into Pgp is determined by autoradiography and phosphorimager analysis. As shown in Figure 3 A; the extent of Vi-induced [$\alpha$-$^{32}$P]8-azidoADP trapping clearly varies in the presence of Pgp substrates. This drug effect is unlikely to be the result of inefficient UV cross-linking or populations of inactive Pgp due to the observed correlation between the extent of Vi-induced 8-azidoADP trapping and the fold-stimulation of the ATPase activity in the presence of a given substrate as discussed below. In addition, the observed variation in the extent of Vi-induced [$\alpha$-$^{32}$P]-8-azidoADP trapping in the presence of various drugs in Figure 3A is not due to unequal loading of protein per lane in the gel (see the immunoblot in Figure 3B; samples in lanes 2-8 in Figure 3A were used for the immunodetection of Pgp with the monoclonal antibody, C219). Only one report suggests this variance (43) while other reports suggest no effect of drug (18,44). [$\alpha$-$^{32}$P]-8azidoADP is incorporated into 2-5% of the Pgp in the reaction depending on the Pgp drug substrates present; these values are consistent with those previously reported for Chinese hamster Pgp in the presence of verapamil (45). It should be noted that cross-linking is highly specific using purified, reconstituted Pgp; the total protein profile of the proteoliposomes used in these studies is also shown in Figure 3, lane 1, having been overloaded to identify impurities. Furthermore, no [$\alpha$-$^{32}$P]-8-azidoADP trapping occurs in the absence of Vi or if the reaction is performed at 4°C a temperature at which, 8-azidoATP (or ATP) is not detectably hydrolyzed by Pgp (data not shown).

The affinity ($K_m$) for 8-azidoATP during vanadate-induced 8-azidoADP trapping is not affected by verapamil and cyclosporin A. We show above that Pgp substrates stimulate trapping
of 8-azidoADP in the Pgp•ADP•Vi transition-state complex (Figure 3 A). To quantitatively compare the effect of substrates on the ATPase activity and the extent of trapping, it is necessary to obtain the kinetic parameters for 8-azidoADP trapping into Pgp. Generating the Pgp•[α-32P]-8azidoADP•Vi complex in the presence of increasing concentrations of [α-32P]-8azidoATP and Vi and quantifying the radioactivity in the Pgp allows one to determine the Km and Vmax (extent of trapped 8azidoADP) of [α-32P]-8azidoATP during trapping. We selected verapamil and cyclosporin A to assess the effect of agents, which either increase or decrease the ATPase activity of Pgp, respectively. It is clear from the data in Figure 4 that the Vmax for [α-32P]-8azidoATP increases in the presence of verapamil and is reduced in the presence of cyclosporin A. The Km (25 ± 7 µM, 23 ± 6 µM and 19 ± 5 µM under basal conditions, in the presence of 50 µM verapamil and 10 µM cyclosporin A, respectively) is, however, not altered by the presence of verapamil or cyclosporin A. As the Km for [α-32P]-8azidoATP was ~20 µM in the presence of Vi, subsequent experiments to determine the extent of trapping in the presence of drugs were performed in the presence of 50 µM [α-32P]-8azidoATP. It is worth noting that the affinity of 8-azidoATP (or ATP) for Pgp is significantly increased in the presence of Vi (Km = 20 µM) when compared to the control (Km = 0.5 mM, Table 2). How Vi alters the affinity of nucleotides for Pgp is not clear at present.

As previously reported (19,25), the Pgp•Mg•8-azidoADP•Vi complex, trapped using Mg•[α-32P]-8azidoATP, dissociates rather quickly (t1/2 = 8 min at 37°C). However, replacing MgCl2 with CoCl2 displays similar reaction kinetics within the first 2 minutes while no dissociation of the Pgp•Co•8-azidoADP•Vi complex is seen up to 20 minutes (data not shown). While Co2+ supports steady-state Pgp ATPase activity at rates 10 to 20-fold slower than Mg2+.
(Kerr and Ambudkar, unpublished data; (19)), for the purpose of Vi trapping experiments, the
divalent cations appear to be equivalent.

The extent of Vi trapping with 50 µM \([\alpha^{32}\text{P}]\)-8azidoATP and 5 mM CoCl\(_2\) in the
presence of various Pgp drug substrates strongly correlates with the fold-stimulation of ATPase
activity by those drugs in the steady-state reaction with saturating Mg\(^{2+}\)•ATP concentrations
(Figure 5; the data used for this analysis are given in Figure 3 A and Table 1, respectively). This
correlation is also seen using higher \([\alpha^{32}\text{P}]\)-8azidoATP concentrations (up to 500 µM) for Vi-
induced trapping. In addition when CoCl\(_2\) was replaced with 5 mM MgCl\(_2\), a similar correlation
between Vi-induced \([\alpha^{32}\text{P}]\)-8azidoADP trapping and the fold-stimulation of ATPase activity
was also observed (data not shown).

The experiments described above clearly demonstrate that drugs, which are substrates of
Pgp affect both ATP-hydrolysis and the Vi-induced trapping of \([\alpha^{32}\text{P}]\)-8azidoATP. This raises
the question as to whether the substrates influence nucleotide binding or a subsequent step during
hydrolysis. We addressed this issue directly by allowing \([\alpha^{32}\text{P}]\)-8azidoATP to bind Pgp under
non-hydrolysis conditions (at 4°C) both in the absence and presence of verapamil or cyclosporin
A. We observed that even saturating concentrations of verapamil and cyclosporin A do not
affect nucleotide binding per se, suggesting that the substrate must act at a step or steps that
follow binding of nucleotide (Sauna, Z. E. and Ambudkar, S.V., manuscript in preparation).
DISCUSSION

For kinetic studies of a membrane transport protein, such as Pgp, large quantities of purified protein which retains complete biological activity is necessary. The baculovirus expression system used in this study produces human Pgp at a high level in the absence of cytotoxic drug selection and the absence of such selection pressure facilitates the assessment of the intrinsic properties of human Pgp. Since Pgp is an integral membrane protein, lipids play a key role in protein conformation and activity (13-15,46). Solubilization and purification in the presence of octyl glucoside and reconstitution with lipid mixture containing bulk E. coli phospholipids, phosphatidylcholine, phosphatidylserine and cholesterol provides a system for the complete recovery of the ATPase activity of purified Pgp (8,11,26).

The ATPase activity of Pgp in the presence of various Pgp drug substrates can be accurately measured using purified, reconstituted Pgp in the cycling assay. In contrast, crude membrane preparations produce high background activities both in the absence of ATP due to NADH oxidases and in the presence of ATP and Vi due to the presence of Vi-insensitive ATPases. Since the cycling assay relies on the catalysis of other enzymes, we demonstrated that compounds in the assay buffer, such as Vi or Pgp drug substrates, do not affect the activity of these enzymes. Furthermore, the slow catalysis of Pgp (approximately 2-10 sec⁻¹) with respect to cycling enzymes, pyruvate kinase and lactate dehydrogenase is crucial to retain the tight coupling from ATP hydrolysis to NADH oxidation.
ATPase activity of Pgp is measured as the Vi-sensitive activity in the presence and absence of various Pgp substrates. The observed variability in the specific activity of Pgp upon reconstitution is likely due to preparation impurities and/or efficiencies of reconstitution; due to these variations, the ATPase activity of Pgp in the presence of drug substrates is typically considered using fold-stimulation values which remain constant. The actual concentrations at which Pgp drug substrates stimulate ATP activity are unclear since all Pgp drug substrates are hydrophobic and partition into the membrane lipid surrounding Pgp at unknown local concentrations. However, characterizing drug-stimulated activities in this purified, reconstituted system is useful for comparisons among various drugs.

The determination of Michaelis-Menten parameters for various Pgp drug substrates is challenging. High amounts of Pgp are required to quantify the enzyme activity in the presence of the drug, and this amount of Pgp sets a lower limit on the amount of drug to be used for stimulation. Hence, many values for \( K_{app} \), as shown in Table 1, are upper limits; however, the substrate-saturated ATPase activities in the presence of these drugs are well defined. Conversely, Pgp drug substrates are highly hydrophobic with low solubility limits making saturation at high drug concentrations problematic. For these drugs, both \( K_{app} \) and drug-saturated ATPase activities are harder to define. Of particular interest is the ability of cyclosporin A and other Pgp “inhibitors” to support ATPase activity. It is clear from the data presented here that cyclosporin A, its analog PSC833, and rapamycin act as Pgp substrates, albeit at a slower ATPase rate than basal levels (Fig. 2A and Table 2). The idea that these drugs act as substrates promoting ATPase activity is supported by several cyclosporin A transport studies (47-50). Additionally, human Pgp-specific monoclonal antibody, UIC2 shift assays suggest similar
conformational changes with vinblastine, a common Pgp substrate, as well as cyclosporin A (51). These studies also demonstrate that the cycling assay provides a useful tool to assess whether a given modulator is a "true" inhibitor or a substrate of Pgp.

It is clear that the ATPase activity of Pgp is stimulated by a myriad of drug substrates. The number and interactions of the drug binding site(s) of Pgp has been discussed extensively in the literature (29,31-34,52,53). While these drugs have profound effects on the overall stimulation of the ATPase activity of Pgp, they have no effect on the values of $K_m$ for nucleotide substrates, ATP and 8-azidoATP (see Fig. 4 and Table 2). Furthermore, the value of $K_i$ for ADP, a competitive inhibitor of ATP, remains constant in the presence of verapamil (Table 2). Taken together, these data suggest that drug substrates, like verapamil and cyclosporin A, have no effect on ATP binding to Pgp; these conclusions are comparable to those suggested using mouse Pgp ($mdr1b$) (20).

The proposed scheme for the catalytic cycle of Pgp and the rate-limiting step is given in Figure 6 (see the figure legend for the detailed description). To investigate other steps of the Pgp catalytic cycle where drug might have an effect, we used Vi-induced ADP trapping experiments, a common method to investigate the transition state(s) of ATP-hydrolyzing proteins without covalent phosphoenzyme intermediates. Ortho-Vi can mimic $P_i$ and bind in its place immediately after ATP hydrolysis and $P_i$ release to trap ADP and inhibit the steady-state turnover of the enzyme (Figure 6, step 5) (18). Therefore, the amount of ADP trapped on Pgp is indicative of the Vi-inhibited conformation, which is comparable to the transition state conformation Pgp•ADP•$P_i$ of the ATP hydrolysis reaction. In this study, the substitution of
CoCl₂ for MgCl₂ avoided complications with dissociation of the Pgp•8-azidoADP•Vi trapped complex while supporting apparently the same rate of reaction for the first two minutes of trapping (data not shown).

Although Vi inhibits all Pgp activity in steady-state experiments independent of the presence of drug substrates, different amounts of 8-azidoADP are trapped on the protein when the extent of Vi–induced trapping is complete. A striking correlation exists between the extent of Vi trapping and the steady-state fold-stimulation of ATPase activity in the presence of various drug substrates. Drugs that support a higher fold-stimulation of steady-state ATPase activity also demonstrate a higher extent of 8-azidoADP (or ADP) trapping; the opposite holds true for less active Pgp drug substrates (see Figure 5). Directly correlating effects on the Vi-induced ADP trapped conformation and the steady-state reaction rates in the presence of Pgp drug substrates indicate that these experiments are measuring the same step in the Pgp catalytic cycle. The Vi-induced ADP trapping experiment measures the amount of ADP released (the inverse of the amount of ADP trapped) from the transition state conformation which, by its correlation, suggests that the rate-limiting step measured in the steady-state reaction is the release of ADP (step 4 or 6 in Figure 6).

These experiments further indicate that the release of Pᵢ is not likely to be the rate-limiting step of the overall catalytic cycle. The low affinity of Pᵢ for Pgp makes this step an unlikely candidate to act as the rate-limiting step of the Pgp reaction cycle (Kᵢ ~30 mM for Vi-induced 8-azidoADP trapping in Pgp [our unpublished data], and Kᵢ ~200 mM for ATP hydrolysis (19)). Additionally, the correlation between the steady state reaction (fold-
stimulation) and the amount of ADP trapping in the presence of drug substrates like verapamil and cyclosporin A indicate that the rate-limiting step is a step after Vi binds and traps Pgp, and P_i release is a prerequisite for Vi binding (Figure 6, step 3 and 5).

The ADP release being the rate-limiting step in the catalytic cycle is further supported by our recent observation that there is an inverse relationship between ADP release from the Pgp•MgADP•Vi complex and the recovery of the substrate binding to the transporter following the transition-state step (25). In addition, the rate of the release of 8-azidoADP (or ADP) from the Vi-trapped Pgp is not affected by the addition of excess nucleotides such as ATP, ADP, or AMPPNP (Sauna, Z.E. and Ambudkar, S.V., unpublished data). It is now clear that most ABC transporters catalyze Vi-sensitive ATP hydrolysis, which is stimulated by substrates. Vi-induced 8-azidoADP trapping has been demonstrated in other transporters such as MRP1 and ABCR (54,55). It is perhaps most likely that the ADP release is a rate-limiting step in the catalytic cycle of other members of the super family of ABC transporters.

ACKNOWLEDGEMENTS

We thank Drs. Michael Gottesman and Ira Pastan for helpful discussions and encouragement and John Gribar and Melissa Smith for comments on the manuscript.
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Table 1. Effect of Selected Drug Substrates on the ATPase Activity of Pgp. Pgp hydrolyzes ATP in the absence of drug substrates at a constant basal rate (fold-stimulation = 1.0). $K_{app}$ represents the drug concentration at half-maximal stimulation of ATPase activity; fold-stimulation represents the ratio of the drug-stimulated $V_{max}$ to the basal $V_{max}$. All assays were performed at saturating Mg•ATP concentrations (5-7.5 mM) using the cycling assay at 37°C to monitor the ATPase activity as described in the Experimental Procedures.

| Drug               | $K_{app}$ (µM) | Fold-Stimulation |
|--------------------|----------------|------------------|
| Progesterone       | >115           | >5               |
| Prazosin           | 16±6           | 4.5              |
| TPP+               | >35            | >3               |
| Valinomycin        | <0.5           | 2.9              |
| Verapamil          | 4.7±1.3        | 2.0              |
| Basal              | NA             | 1.0*             |
| Cyclosporin A      | <2             | 0.5              |
| PSC833b            | <2             | 0.4              |
| Rapamycin          | <4             | 0.2              |

NA, not applicable

* As defined in the legend

$^a$ Tetraphenylphosonium ion, from the chloride salt

$^b$ PSC833 is a cyclosporin A analog
Table 2. Michaelis-Menten Parameters for Nucleotide Hydrolysis by P-glycoprotein in the Presence of Selected Agents.

Values of $K_m$ for ATP and 8-azidoATP and $K_i$ for ADP were determined in the presence and absence of saturating Pgp substrates representing ATPase activities both greater and less than basal activity (verapamil, 50 µM; cyclosporin A, 10 µM; all assays contained 1% DMSO). Kinetic analyses were carried out as described in the Experimental Procedures.

| Nucleotide | Drug Substrate       | $K_m$ ATP (mM) | $V_{max}$ (nmol min$^{-1}$ mg$^{-1}$) | $K_i$ ADP (mM) |
|------------|----------------------|----------------|-----------------------------------|----------------|
| ATP$^a$    | Verapamil            | 0.22 ± 0.03    | 1150                              | NA             |
| No drug (basal)$^b$ |             | 0.33 ± 0.04    | 480                               | NA             |
| Cyclosporin A |                 | 0.26 ± 0.02    | 233                               | NA             |
| 8-azidoATP$^c$ | Verapamil        | 0.4 ± 0.08     | 144                               | NA             |
| No drug (basal)$^b$ |             | 0.6 ± 0.15     | 62                                | NA             |
| Cyclosporin A |                 | 0.5 ± 0.10     | 37                                | NA             |
| ADP$^c$    | Verapamil            | NA             | NA                                | 0.5$^d$ ± 0.2  |
| No drug (basal)$^b$ |             | NA             | NA                                | 0.3$^d$ ± 0.1  |

NA, not applicable

$^a$ determined using the ATPase cycling assay

$^b$ basal activity assayed in the absence of drug substrates as described in Experimental Procedures
c determined using the ATPase $P_i$ release (end point) assay

d competitive inhibition pattern
FIGURE LEGENDS:

Figure 1: ATPase Activity of Purified, Reconstituted Human P-glycoprotein using the Cycling Assay. Each assay contained 5-10 µg Pgp in ATPase assay buffer containing 15 mM MgCl₂ and cycling components with (O) or without (◊) 50 µM verapamil. Closed symbols represent non-Pgp ATPase activity in the presence of 0.3 mM Vi. The ATPase activity was measured by monitoring the oxidation of NADH at OD₃₄₀nm as described in Experimental Procedures.

Figure 2: Effect of Selected Substrates on the ATPase Activity of P-glycoprotein. A. ATPase activity of purified, reconstituted Pgp (5-10 µg protein) was assayed with 7 mM ATP, 14 mM MgCl₂, and various concentrations of verapamil (●) or prazosin (▲). Activity is shown on the y-axis as nmol of ATP hydrolyzed•min⁻¹•mg⁻¹. The inset in A demonstrates the saturated ATPase activity in the presence of indicated concentrations (µM) of cyclosporin A (●)(CSA). B. The ATPase activity of Pgp was measured with 14 mM MgCl₂ and various concentrations of ATP ranging from 0.05 to 7.5 mM in the presence of 50 µM verapamil (●), 10 µM cyclosporin A (▲), or in the absence of added drug (basal) (■). Data in A and B were fit as described in Experimental Procedures; the dashed line in the inset in panel A is not a curve fit.

Figure 3: Vanadate-induced 8-azidoADP Trapping in Pgp the Presence of Selected Agents. (A) Purified, reconstituted Pgp (17 µg) was subjected to SDS-PAGE and stained as described in Experimental Procedures (lane 1). The protein is overloaded to emphasize impurities (the
molecular masses in kilodaltons are indicated on the left). Lanes 2-8 show the effect of selected agents on the extent of Vi-induced trapping of [α-32P]-8azidoADP. Reconstituted Pgp (0.5 mg/ml) was incubated with 50 µM [α-32P]-8azidoATP (5-7.5 µCi/nmole) in ATPase assay buffer containing 5 mM CoCl2, and 0.3 mM Vi for 15 min at 37°C in the presence of indicated drug substrate. The reaction was quenched by addition of excess ATP (12.5 mM) at 4°C, UV-cross-linked AT 365 nm, run on SDS-PAGE, and subjected to autoradiography and phosphorimager analysis as described in *Experimental Procedures*. In lanes 2-8, same amount of protein (10 µg) was loaded in each lane. Lane 2, the reaction was quenched 10 seconds after the addition of [α-32P]-8azidoATP (control); lane 3, rapamycin (20 µM); lane 4, cyclosporin A (1 µM); lane 5, basal (only DMSO); lane 6, verapamil (50 µM); lane 7, valinomycin (5 µM); and lane 8, prazosin (100 µM). (B) An immunoblot of protein samples in lanes 2-8 in (A). The immunodetection of Pgp by using the monoclonal antibody, C219 was carried out on samples in lanes 2-8 in A (0.5 µg protein/lane) as described under *Experimental Procedures*.

**FIGURE 4: Determination of K_m for [α-32P]-8azidoATP during Vanadate-induced 8-azidoADP Trapping in Pgp in the Presence of Verapamil and Cyclosporin A.**

Purified Pgp, reconstituted into proteoliposomes (20 µg) was incubated in the ATPase reaction mixture in the absence of drugs (■) and in the presence of 50 µM verapamil (●) or 10 µM cyclosporin A (▲) at 37°C for 3 min. Indicated concentrations of [α-32P]-8azidoATP (1.0 to 75 µM; 5-10 µCi/nmole) were then added in the dark and the samples were incubated for an additional 10-min at 37°C. The reaction was stopped by the addition of 12.5 mM ice-cold ATP and placing the samples on ice. The samples were photocrosslinked by UV irradiation at 365 nm.
at 4°C. Following SDS-PAGE on an 8% gel (10 µg protein/lane), radioactivity in the Pgp band was estimated using a STORM 860 phosphorimager system.

**Figure 5: Correlation between the Extent of Vanadate-induced 8-azidoADP Trapping and the Fold-Stimulation of ATPase Activity in the Presence of Pgp Substrates.** The extent of Vi-induced trapping of [α$^{32}$P]-8azidoADP (values from phosphorimager analysis as in Figure 3) after a 15 min incubation at 37°C in the presence of 50 µM [α-$^{32}$P]-8azidoATP and 5 mM CoCl$_2$ was correlated ($r^2=0.941$) to the fold-stimulation of ATPase activity under saturating MgATP conditions as shown in Figure 2B and Tables 1 and 2, respectively. The numbers represent the data obtained in presence of indicated agent: 1, rapamycin (20 µM); 2, cyclosporin A (1 µM); 3, DMSO; 4, verapamil (50 µM); 5, valinomycin (5 µM); and 6, prazosin (100 µM). A similar correlation between Vi-induced [α-$^{32}$P]-8azidoADP trapping and the fold-stimulation of ATPase activity was also observed when CoCl$_2$ was replaced with 5 mM MgCl$_2$ (data not shown).

**Figure 6: Proposed Scheme for the Catalytic Cycle of Pgp.** The proposed scheme is similar to those previously described (20,22,29) and shows the rate-limiting step (Step 4) in the catalytic cycle based on the work reported here. Step 1: Drug substrate and ATP bind to Pgp. There is no evidence that the binding of one is either a prerequisite or inhibitory to the binding of the other. Step 2: Binding of drug and ATP is followed by ATP-hydrolysis and this is accompanied by a conformational change resulting in the translocation of the drug from a high-affinity (ON) to a low affinity (OFF) site. Step 3: Following hydrolysis of ATP, both P, and drug are released although the exact order of release is not known at present. Step 4: The ADP release, which appears to be the slowest step in the cycle (see below), is essential for regenerating Pgp for the
next hydrolysis event (the box is shaded to indicate the rate-limiting nature of this step). Step 5: If Vi is provided to the system, Vi mimics \( P_i \) to trap ADP in a stable ternary conformation (\( Pgp\cdot ADP\cdot Vi \). Moreover, given the chemical analogy between \( P_i \) and Vi, the general consensus is that the \( Pgp\cdot ADP\cdot P_i \) and the \( Pgp\cdot ADP\cdot Vi \) complexes are equivalent and that this transition-state represents an intermediate state during the normal reaction pathway (56). Step 6: Eventually, Vi and ADP dissociate from Pgp (\( t_{1/2} = 80-90 \text{ min at } 37^\circ C \)) to initiate the next cycle. The observed correlation between the extent of trapped 8-azidoADP in the presence of Vi and fold-stimulation of ATP hydrolysis by various substrates (see Fig. 6), and the fact that \( P_i \) exhibits very low affinity for Pgp (\( K_i \sim 30 \text{ mM for Vi-induced 8-azidoADP trapping in human Pgp (our unpublished data) and } K_i \sim 200 \text{ mM for ATP hydrolysis by Chinese hamster Pgp (19) strongly suggest that 8-azidoADP (ADP) release (step 4) is the rate-limiting step in the catalytic cycle and substrates modulate the Pgp activity by exerting effect on this step. Although not shown, both ATP and ADP are complexed with Mg\(^{++}\), which has been omitted for clarity.}
FIGURE 1
FIGURE 2A

ATP hydrolysis (nmol·min⁻¹·mg⁻¹)

Drug [µM]

[CSA], µM

0 10 20 30 40 50

0 175 350 525 700

0 25 50 75 100 125

0 2 4 6 8 10
FIGURE 2B

ATP hydrolysis (nmol·min⁻¹·mg⁻¹)

[ATP], mM

0 1 2 3 4 5

0 400 800 1200

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FIGURE 4

[α^{32}P] 8AzidoATP [µM] vs. [α^{32}P] 8AzidoADP incorporation (arbitrary units)
FIGURE 5

[\alpha^{32}P]AzidoADP incorporated (arbitrary units) vs. fold-stimulation
FIGURE 6
Correlation between steady-state ATP hydrolysis and vanadate-induced ADP trapping in human P-glycoprotein: Evidence for ADP release as the rate limiting step in the limiting step in the catalytic cycle and its modulation by substrates
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J. Biol. Chem. published online December 19, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M010044200

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