Complete Inhibition of the Pdr5p Multidrug Efflux Pump ATPase Activity by Its Transport Substrate Clotrimazole Suggests that GTP as Well as ATP May Be Used as an Energy Source†

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ABSTRACT: The yeast Pdr5p transporter is a 160 kDa protein that effluxes a large variety of xenobiotic compounds. In this study, we characterize its ATPase activity and demonstrate that it has biochemical features reminiscent of those of other ATP-binding cassette multidrug transporters: a relatively high K_m for ATP (1.9 mM), inhibition by orthovanadate, and the ability to specifically bind an azidoATP analogue at the nucleotide-binding domains. Pdr5p-specific ATPase activity shows complete, concentration-dependent inhibition by clotrimazole, which is also known to be a potent transport substrate. Our results indicate, however, that this inhibition is noncompetitive and caused by the interaction of clotrimazole with the transporter at a site that is distinct from the ATP-binding domains. Curiously, Pdr5p-mediated transport of clotrimazole continues at intracellular concentrations of substrate that should eliminate all ATPase activity. Significantly, however, we observed that the Pdr5p has GTPase and UTPase activities that are relatively resistant to clotrimazole. Furthermore, the K_m(GTPase) roughly matches the intracellular concentrations of the nucleotide reported for yeast. Using purified plasma membrane vesicles, we demonstrate that Pdr5p can use GTP to fuel substrate transport. We propose that Pdr5p increases its multidrug transport substrate specificity by using more than one nucleotide as an energy source.

The ATP-binding cassette (ABC)[1] superfamily of transporters is one of the largest families of proteins and has been found to occur in all organisms that have been studied (*1*, *2*). The first eukaryotic ABC transporter identified, P-glycoprotein (P-gp, ABCB1), was recognized to have enormous clinical relevance as it was implicated in multidrug resistance (MDR) in human cancers (*3*). ABC transporters are now recognized to play a central role in biology, and mutations in 17 human ABC proteins are implicated in human diseases (*2*). MDR is a significant impediment not only to effective cancer chemotherapy but also in the treatment of infectious diseases such as those caused by pathogenic yeast species (*4*). The yeast ABC transporter, Pdr5p, which is a functional ortholog of human P-gp, mediates resistance to a broad array of structurally and mechanistically diverse xenobiotic compounds (*5*). Although Pdr5p is probably the most extensively studied ABC transporter in *Saccharomyces cerevisiae*, the biochemistry of yeast ABC proteins is poorly understood compared to that of their mammalian counterparts.

The basic unit of an ABC protein is a transmembrane domain (TMD) (generally composed of six α-helices) and an intracellular nucleotide-binding domain (NBD). The functional unit is composed of two TMDs and two NBDs. Pdr5p exhibits the architecture of a typical ABC protein, but the order of the NBDs and TMDs is reversed vis-à-vis P-gp (*6*). Transport mediated by ABC transporters involves coupling the energy of ATP hydrolysis to mechanical movements at the transport substrate sites (*7*). Thus, the catalytic cycle of ATP hydrolysis has been extensively studied (for reviews, see refs *7*−*9*). In previous studies, the ATPase activity of Pdr5p was characterized in purified plasma membrane vesicles and proteoliposomes (*10*). The activity in the latter was extremely low, and the former contained additional, as yet unidentified transporters that contaminated the preparation; e.g., the *pdr5::Tn5*-null mutant strain retained considerable ATPase activity. Furthermore, although a very small stimulation of NTPase activity with cycloheximide was observed, extensive analysis of the effect of Pdr5p substrates on ATPase activity was not carried out. Recently, the ATPase activity of purified Cdr1p from *Candida albicans*, which is a homologue of Pdr5p, has been characterized (*11*, *12*). The Cdr1p-ATPase displays a high K_m for ATP (1.8−2.1 mM), and drug substrates moderately

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[1] Abbreviations: ABC, ATP-binding cassette; BeF_x, beryllium fluoride; R6g, rhodamine 6G; chl, chloramphenicol; 5FOA, 5-fluoroorotic acid; IAAP, iodoarylazidoprazosin; NBD, nucleotide-binding domain; P-gp, P-glycoprotein; Vi, orthovanadate.

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Table 1: Yeast Strains Used in This Study

strain	genotype	origin
AD124567	*MAT*α *his3*, *ura3*, Δ*pdr3*, Δ*ycf1*, Δ*yor1*, Δ*pdr10*, Δ*pdr15*, Δ*snq2*	Dr. A. DeCottignies, 5 FOA *ura3* derivative made by J. Golin
AD1-7	identical to AD124567 but Δ*pdr5*	Dr. A. DeCottignies, 5 FOA derivative made by J. Golin
JG2000	identical to AD1-7, but containing pSS607 (*PDR5*)	this study
JG2002	identical to JG2000, but cured of pSS607 by 5FOA treatment; retains Δ*pdr5*	this study

stimulate its activity. Even though hydrolysis of NTPs other than ATP has been reported for some ABC proteins (*12*), the functional significance vis-à-vis transport has not been addressed.

The potent Pdr5p transport substrates clotrimazole and tritylimidazole can be exploited in improving our understanding of the coupling between NTPase activity and transport of drug substrate. These xenobiotic compounds define biochemically separable sites involved in drug transport that may overlap (*5*, *13*). Furthermore, clotrimazole and its related derivatives are clinically significant compounds. They are antifungal agents and also reduce the level of red blood cell dehydration by inhibiting a water/K^+ channel (*14*). The latter makes them candidate drugs in the treatment of sickle cell anemia. Clotrimazole also shows mixed, incomplete inhibition of the sarcoplasmic reticulum calcium transporter ATPase (*15*).

In this study, we demonstrate ATPase activity that can clearly be attributed to Pdr5p, is orthovanadate (Vi)-sensitive, and shows Henri−Michaelis−Menten kinetics. A surprising result was that clotrimazole completely inhibited ATPase activity in vitro with an IC_{50} of ~2.5 μM. However, transport in vivo continues even in the presence of what should be inhibitory concentrations of clotrimazole. It has been known for some time that in contrast to most of the other ABC drug transporters in yeast, Pdr5p has significant NTPase activity (*10*). We determined the kinetic parameters for GTPase and UTPase activity. The K_m(UTPase) was high, suggesting that this particular activity has little if any physiological role. The K_m(GTPase), however, is roughly the same as the intracellular concentration of the nucleotide. Furthermore, this activity is considerably more resistant to clotrimazole than is the ATPase activity. Finally, we demonstrate that Pdr5p can use GTP to fuel the uptake of [^{3}H]chloramphenicol in plasma membrane vesicles. Taken together, these data suggest that Pdr5p may increase its substrate specificity by using more than one type of nucleotide as an energy source.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media. The yeast strains used in this study are found in Table 1. AD1-7 and AD124567 were previously described (*6*). AD124567 overexpresses Pdr5p and is otherwise isogenic to AD1-7, which lacks all major plasma membrane ABC transporters and is called Δ*pdr5* here. A *ura3* AD1-7 derivative was made by negative selection on 5-fluoroorotic acid (5FOA) as described elsewhere (*16*). Plasmid pSS607 was constructed from pGL706 (*17*) by digesting the latter with *Spe*I to remove ~2 kb of the 2μ circle origin of replication and religating it with T4 DNA ligase. As a result, an integrative plasmid was created. This construction was used to transform AD1-7 and thus create a new, stable Pdr5p-overproducing strain, JG2000. To do this, lithium acetate transformation of yeast with pSS607 was carried out with a Gietz transformation kit (Medicorp, Montreal, QC). The construction of JG2002 is described in the Results. To grow cells for membrane vesicle preparation, strains were cultured in up to 2 L of YEPD medium until they reached a concentration of $2-4 \times 10^7$ cells/mL before being processed as previously described (*13*). Growth was monitored with a Jenway (Essex, England) 6105 UV−vis spectrophotometer.

Chemicals. The xenobiotic compounds were dissolved in DMSO. All of the nonradioactive reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO). We purchased 8-azido[α-^{32}P]ATP (15−20 Ci/mmol) and 8-azido-[α-^{32}P]ADP (15−20 Ci/mmol) from Affinity Labeling Technologies, Inc. (Lexington, KY). We purchased [^{3}H]-clotrimazole (8 Ci/mmol) for the experiments described in the legend of Figure 3 from Moravek Radiochemicals (Brea, CA). For those experiments described in the legend of Figure 6C, we obtained [^{3}H]clotrimazole (20 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]-Chloramphenicol (20 Ci/mmol) was also obtained from the latter source.

Determination of the Minimum Inhibitory Concentration (MIC) of Clotrimazole. The MIC of clotrimazole was determined for the Pdr5p and Δ*pdr5* strains as previously described (*5*). Plates were incubated for 96 h prior to scoring.

Spot Tests of Drug Sensitivity. The relative sensitivity of strains was qualitatively tested by varying the cell concentration in a 5 μL volume. Overnight cultures of strains were washed with sterile water. Following determination of the cell concentration, serial dilutions were carried out so that the number of cells spotted onto a plate in 5 μL varied from 10^4 to 5×10^6. Plates were incubated for 72 h before being photographed.

Preparation of Purified Membrane Vesicles. The procedure for the preparation of purified yeast plasma membrane vesicles was initially described by Shukla et al. (*18*) with minor modifications described recently (*13*). When vesicles were used for transport (uptake) experiments, they were resuspended in the transport buffer described below. The protein concentration in the vesicles was determined with a bicinchonic acid kit (Perbio, Rockland, IL).

Assay of ATPase Activity. The ATPase activity was assayed as previously described (*18*) in an ATPase buffer containing 100 mM MOPS (pH 7.4), 50 mM KCl, 5 mM NaN_3, 2 mM EGTA (pH 7.0), 2 mM DTT, and 10 mM $MgCl_2$ at 30 or 35 °C. We determined that Pdr5p activity was sensitive to 300 μM Vi; thus, the Pdr5p-specific activity was taken as the Vi-sensitive component and used in all calculations. Assay buffer, water, Vi, and all inhibiting substrates were mixed at room temperature. Following addition of purified plasma vesicles, samples were incubated at 30 or 35 °C for 8 min. After this, the reaction was initiated by addition of ATP (or another nucleotide). Reactions were terminated by addition of SDS to a final concentration of 2.5%. Analysis of enzyme kinetics was performed with GraphPad (San Diego, CA) Prism.

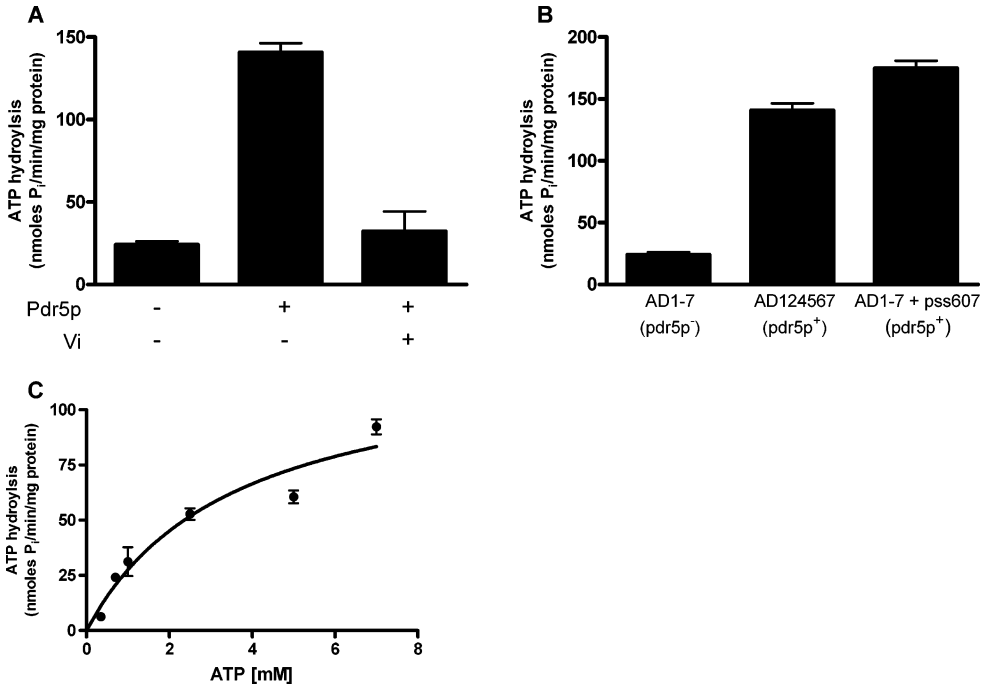

FIGURE 1: ATPase activity of Pdr5p. The ATPase assay was carried out as described in Experimental Procedures at 35 °C. The mean values are plotted, and the error bars represent the standard deviation ($n = 3$). (A) The sensitivity of Pdr5 ATPase to Vi (0.3 mM) was measured at 35 °C with 5 mM ATP. Analogous results were also obtained at 30 °C (data not shown). (B) Vi-sensitive ATPase activity was measured in purified membrane vesicles (10 μg/assay) prepared from yeast strains AD1-7 (Pdr5p$^-$), AD124567 (Pdr5p$^+$), and AD1-7 with pSS607 (Pdr5p$^+$). Recovery of Pdr5p ATPase activity in the Δ*pdr5* mutant was achieved by lithium acetate transformation of this strain with pSS607. ATPase assays were carried out in the presence of 5 mM ATP. (C) Vi-sensitive ATPase activity was measured in purified membrane vesicles (10 μg/assay) prepared from yeast strain AD124567 (Pdr5p$^+$) in the presence of the indicated concentrations of ATP. The data were fit to the Henri−Michaelis−Menten equation using GraphPad Prism.

[^{3}H]Clotrimazole Transport Assay. A steady-state, 2-deoxyglucose-sensitive assay was used to assess Pdr5p-mediated transport of [^{3}H]clotrimazole (0.6−25 μM). The assay is similar to one previously described for chloramphenicol (*5*) except that the incubation time is 90 min instead of 3 h and the washed pellets were recovered by centrifugation rather than by filtration.

Photoaffinity Labeling of Pdr5p with 8-Azido[α-^{32}P]ATP. To monitor binding of 8-azido[α-^{32}P]ATP to Pdr5p, plasma membranes (40 μg/assay) were suspended in MOPS ATPase assay buffer (see above) containing 50 μM 8-azido[α-^{32}P]-ATP (2.5 μCi/nmol) before addition of either 2 mM ATP or 20 μM clotrimazole and incubated in the dark for 10 min at 4 °C. The effect of clotrimazole on the kinetics of the nucleotide binding assay was modified slightly. Plasma membranes prepared from Pdr5p-expressing cells (40 μg/assay) were incubated with increasing concentrations of 8-azido[α-^{32}P]ADP (10 μCi/nmol) or 8-azido[α-^{32}P]ATP (10 μCi/nmol) in the dark for 10 min at 30 °C either in the presence or in the absence of 20 μM clotrimazole. Following incubation with the nucleotides, the samples were cross-linked on ice with 365 nm UV light for 10 min. Reactions were stopped by adding 12.5 μL of 5× SDS−PAGE sample loading buffer. All samples were then electrophoresed on a Tris-acetate gel at a constant voltage, and the gels were dried. The radioactivity incorporated into the Pdr5p band was quantified with a STORM 860 phosphorimager system (Molecular Dynamics, Sunnyvale, CA) and ImageQuant.

[^{3}H]Chloramphenicol Transport in Vesicles. GTP-dependent transport of [^{3}H]chloramphenicol was assessed using a previously described rapid filtration technique (*19*). Thawed membrane vesicles (25 μg) were incubated at 30 °C for 5 min before being diluted in transport buffer [10 mM Hepes (pH 7.5), 20 mM KCl, 250 mM sucrose, and 10 mM $MgCl_2$] containing [^{3}H]chloramphenicol (20 Ci/mmol) made up to 5.0 μM with cold compound in the presence or absence of 5 mM GTP in a final volume of 55 μL. Samples were incubated at 30 °C. At a specified time, they were diluted in 1 mL of stop buffer (transport buffer without $MgCl_2$) and filtered through Whatman 0.22 μm nitrocellulose filters. The filters were washed with 5 mL of stop buffer before the samples were counted in a scintillation counter.

RESULTS

Pdr5p Exhibits Specific, Vi-Sensitive ATP Hydrolysis and Has a High K_m(ATP). The ATPase activity of ABC proteins expressed in mammalian or insect cells has been extensively characterized (for reviews, see refs *3* and *20*). It has been much more difficult to characterize the ATPase activity of yeast ABC proteins, and study has endeavored to measure only Pdr5p-mediated ATPase activity, although the genetic background we describe below has been used to study the Cdr1p transporter (*21*). One major problem in determining the ATPase activity that can be specifically attributed to Pdr5p is that the plasma membranes of yeast express the proton-transporting form (Pma1p [H$^+$]), an essential gene product, which therefore cannot be eliminated by a deletion mutation. This enzyme appears to be sensitive to only Vi (*22*), which also inhibits ABC proteins. However, the optimal pH for Pma1p [H$^+$] is ∼5.5−6.0, and the enzyme is only minimally active at pH 7.5 (*10*). We therefore characterized ATPase activity in plasma membranes from the Pdr5p and Δ*pdr5* strains at pH 7.5. Figure 1A shows that the plasma membrane vesicles that express Pdr5p exhibit an ATPase

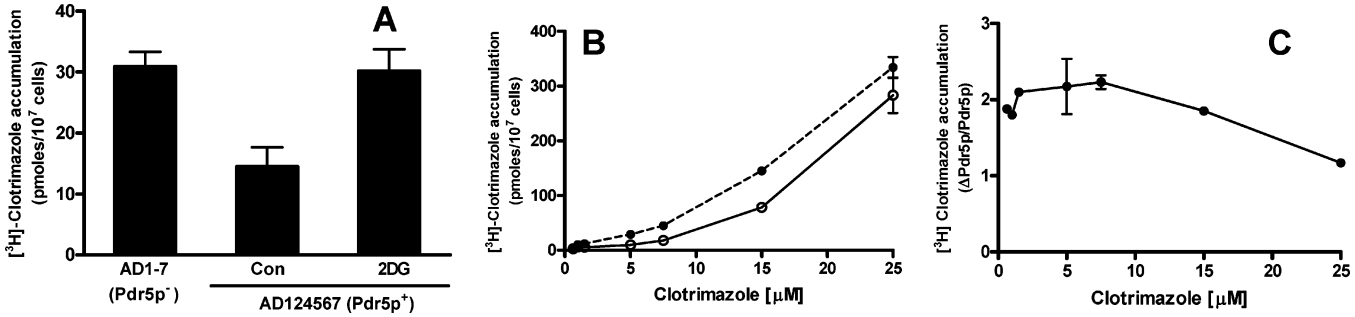

FIGURE 2: Transport of [^{3}H]clotrimazole by Pdr5p under steady-state conditions. Cells (10^7) were incubated in Hepes buffer (pH 7.0) containing 5 μM [^{3}H]clotrimazole (8 Ci/mmol) for 90 min at 30 °C in a total volume of 100 μL. Following this, they were washed with 800 μL of cold 50 mM Hepes (pH 7.0) buffer. Washed pellets were resuspended in 100 μL of Hepes, and the samples were placed in scintillation fluid for counting in a Packard (Meriden, CT) TriCarb liquid scintillation counter. In each transport experiment, the mean values are plotted and the error bars represent the standard deviation ($n = 3$). (A) Energy-dependent accumulation of [^{3}H]clotrimazole (5 μM) was assessed in intact Pdr5p-deficient and -expressing cells. In the Pdr5p-expressing cells, the measurements were taken in the absence and presence of 60 mM 2-deoxyglucose. (B) Steady-state, 2-deoxyglucose-sensitive (60 mM) clotrimazole transport was assayed in Δ*pdr5* and Pdr5p at 0.6, 1.0, 1.5, 5.0, 7.5, 15, and 25 μM clotrimazole at 30 °C as described in Experimental Procedures. The average amount of accumulation (picomoles per 10^7 cells) in three samples was obtained for each strain at each concentration: (○) Pdr5p and (●) Δ*pdr5*. (C) The results are graphed as the ratio of accumulated clotrimazole in AD1-7 (Δ*pdr5*) to that of AD124567 (*PDR5*). As a control, inhibition of transport by 60 mM 2-deoxyglucose was tested at 1.0, 1.5, 5.0, and 7.5 μM clotrimazole.

activity at least 6-fold higher than that of the Pdr5p$^-$ vesicles. Moreover, the ATPase activity of the Pdr5p membranes is sensitive to Vi, with activity comparable to that observed in the Δ*pdr5* membranes. These measurements were taken at pH 7.5 and suggest that the Pma1p [H^+] activity is quite low at pH 7.5 in these membrane preparations. The data also support the view that either the activity of the Δ*pdr5* membranes or Vi-insensitive ATPase activity in the Pdr5p membranes can be considered as background. In addition, when the Δ*pdr5* strain was transformed with *PDR5*-bearing plasmid pSS607 (Figure 1B) to create JG2000, ATPase activity was restored to levels observed in the AD124567 membranes (Figure 1B). Taken together, the data in Figure 1 indicate that we can measure ATP hydrolysis that can be attributed almost solely to Pdr5p. The high, unstimulated ATPase activity of Pdr5p that we report was also noted in the earlier study of its NTPase properties (*10*) and is generally not observed with other yeast ABC drug transporters (*23*). We also found that Vi-sensitive ATP hydrolysis in the Pdr5p membranes is linear for at least 10 min (data not given). Thus, we monitored ATPase activity for 8 min to measure the specific activity (nanomoles of P_i per milligram of purified vesicle protein per minute).

These experiments were all carried out in the presence of 5 mM ATP, a saturating concentration determined from previous reports that the K_m(ATP) for ABC proteins is in the range of 0.3−2.1 mM. To establish the kinetic parameters for Pdr5p-mediated ATP hydrolysis, we measured the Vi-sensitive specific activity of P-gp in the presence of increasing concentrations of ATP. Pdr5p-mediated hydrolysis shows Henri−Michaelis−Menten kinetics with a K_m(ATP) of 1.9 mM at 35 °C (Figure 1C). A highly Pdr5-specific ATPase activity is also observed at 30 °C (a temperature conventionally used for transport assays). The K_m(ATP) was comparable, although the activity at the latter is ~4-fold lower (V_{max} ~ 30 nmol min^{-1} mg^{-1}). Moreover, omission of the magnesium ion from the assay buffer reduces the rate of hydrolysis to the same level observed in Δ*pdr5* or the nonmembrane control (data not given). This is consistent with biochemical evidence that suggests that the presence of a divalent cation such as magnesium is mandatory during hydrolysis by ABC proteins (*24*, *25*) and is consistent with crystal structures of the ATP sites of ABC proteins (*26*, *27*).

Clotrimazole Is Transported by Pdr5p. The catalytic cycles of ABC transporters have been studied in detail, and there appears to be a conformational coupling between transport substrate- and nucleotide-binding sites (for reviews, see refs *28* and *29*). Furthermore, the stimulation or inhibition of ATPase activity by drug substrates suggests that ATP hydrolysis and transport are obligatorily coupled. Clotrimazole is an important antifungal agent and a potent Pdr5p substrate, as measured by the MIC ratio (for a discussion of the MIC ratio, see ref *1*). *PDR5* strains are ~300−1000-fold more resistant than their isogenic Δ*pdr5* counterparts (*5*). We determined the MIC for the two strains used in this study. The Pdr5p strain grew on medium containing 15 μM clotrimazole but not on plates containing 25 μM clotrimazole. The MIC for the isogenic Δ*pdr5* strain, in contrast, is 0.03 μM.

We determined the relative ability of Δ*pdr5* and Pdr5p strains to transport clotrimazole under steady-state conditions, where the drug remains in the reaction mixture (not unlike determining an MIC in liquid or solid medium, where the drug is continually present). We examined concentrations of clotrimazole ranging from 0.6 to 25 μM. Figure 2A shows a representative experiment carried out at 30 °C with 5.0 μM clotrimazole [three independent experiments each ($n = 3$) were carried out with similar results]. Under steady-state conditions, the Pdr5p strain consistently accumulates ~2−2.5-fold less [^{3}H]clotrimazole than the isogenic Δ*pdr5* mutant. The Pdr5p-mediated efflux of clotrimazole appears to depend on ATP hydrolysis because addition of 60 mM 2-deoxyglucose completely inhibits the transport. Similar experiments were carried out at six other concentrations of clotrimazole. These results are shown in Figure 2B. The amount of retained clotrimazole increases as a function of clotrimazole concentration and does not saturate, but the Pdr5p strain retains significantly less compound ($p < 0.001$ for 5.0, 7.5, and 15 μM) at each point except the last (25 μM). A two-way ANOVA of the data indicates that the probability that the mutant versus wild-type lines are the same is <0.0001. Figure 2C shows these data as the ratio of

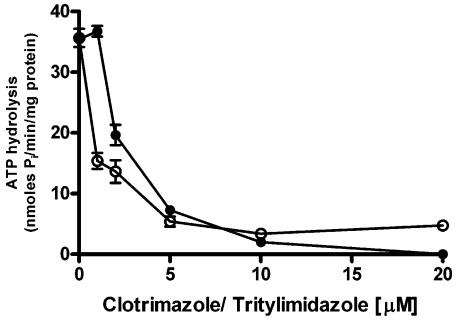

FIGURE 3: Inhibition of Pdr5p ATPase by clotrimazole and tritylimidazole. The ATPase assay was carried out at 35 °C as described in Experimental Procedures, except that varying concentrations of clotrimazole (●) or tritylimidazole (○) were added to the reaction mix containing 5 mM ATP. The mean values are plotted, and the error bars represent the standard deviation ($n = 2$).

the amount of retained [^{3}H]clotrimazole in Δ*pdr5* (where Pdr5p is not expressed) to that in the Pdr5p strain. This ratio provides a measure of the transport capability of Pdr5p at each concentration of the transport substrate. Transport capability in the Pdr5p strain is fairly constant over a 15-fold concentration range of [^{3}H]clotrimazole, until ~15 μM.

We also preloaded cells with 7.5 μM clotrimazole for 90 min, washed them with Hepes buffer to remove [^{3}H]-clotrimazole, and then placed them in Hepes-glucose to efflux substrate for 90 min. Under these conditions, the difference between the strains was 3-fold (Δ*pdr5*, 31.5 ± 7.8 pmol/10^7 cells; Pdr5p, 10.44 ± 1.56 pmol/10^7 cells) and thus not very different from the steady-state conditions that we typically use. Furthermore, previous studies (*5*) conducted with tritylimidazole used an assay that was identical to the one described just above for clotrimazole and gave similar results (a 2.5-fold difference).

Finally, we carried out a parallel steady-state experiment with 2.5 μM rhodamine 6G (R6g). Under loading conditions identical to those used for clotrimazole (steady state, 90 min, 30 °C), the Δ*pdr5* strain accumulated 788.2 ± 109.9 arbitrary units and the Pdr5p counterpart 7.590 ± 1.626. Addition of 60 mM 2-deoxyglucose abolished all R6g efflux (772.58 ± 132.0). The difference between strains is ~100-fold and thus is substantially larger than the one observed with clotrimazole or tritylimidazole. These results strongly demonstrate that the relatively modest differences observed with the two imidazole derivatives are an inherent feature of these substrates and not the particular method of assessing transport.

Inhibition of Pdr5p ATPase Activity by Clotrimazole and Tritylimidazole. Many studies with ABC transporters have demonstrated that transport substrates influence ATP hydrolysis (*3*). We therefore examined the effect of clotrimazole on Pdr5p-mediated ATP hydrolysis. The data in Figure 3 show that clotrimazole inhibited the ATPase reaction in a concentration-dependent manner. The IC_{50}(ATPase) for clotrimazole at 30 °C is ~2.5 μM, and ATPase activity is completely abolished between 15 and 20 μM.

Tritylimidazole had a similar effect on ATPase activity. The data in Figure 3 indicate that this compound has an IC_{50} much like that of clotrimazole. However, unlike clotrimazole, tritylimidazole does not completely abolish ATPase activity. In the experiment shown in Figure 3, there was a residual activity of ~22%. In subsequent experiments carried out at 35 °C, the remaining activity ranged from 25 to 36% (data not shown).

The inhibitory effect of clotrimazole cannot be attributed to direct interaction of clotrimazole and ATP. We measured the amount of P_i released at 35 °C when 5 mM ATP was included in a reaction mix without membranes. We compared the effect of adding 0, 1, 10, and 100 μM clotrimazole. We observed 1.0−1.1 nmole/min in all cases. Furthermore, the ATPase activity of P-glycoprotein measured in crude membranes under similar conditions is not altered by addition of 20 μM clotrimazole (data not given).

Clotrimazole and Tritylimidazole Are Noncompetitive Inhibitors of Pdr5p ATPase Activity. The specific binding of nucleotides at the ATP site(s) is a prerequisite for catalysis, and the photoaffinity analogue of ATP, 8-azido[α-^{32}P]ATP, has been extensively used to probe the nucleotide-binding sites of ABC transporters. We demonstrate that 8-azido[α-^{32}P]ATP binds specifically to the ATP sites of Pdr5p (Figure 4A), because excess ATP inhibits photolabeling completely (compare lanes 9 and 10 of Figure 4A). Moreover, there is no discernible signal when vesicles prepared from Δ*pdr5* cells are used (Figure 4A, lane 7). The photo-cross-linked 8-azido[α-^{32}P]ATP colocalizes with Pdr5p identified on a colloidal blue-stained gel (see lanes 3 and 9). It is important to note that plasma membranes prepared from Δ*pdr5* cells do not show the Pdr5p band in the colloidal blue-stained gel (Figure 4A, lanes 1 and 2), and there is no band photolabeled by 8-azido[α-^{32}P]ATP (Figure 4A, lane 7). We overloaded the Δ*pdr5* samples to conclusively demonstrate that there is neither expression of the Pdr5p in these samples nor cross-linking of 8-azido[α-^{32}P]ATP. The autoradiogram of the Δ*pdr5* mutant does, however, suggest that the partially purified plasma membranes contain proteins other than Pdr5p that bind 8-azido[α-^{32}P]ATP (Figure 4A, lane 7).

The data presented in Figure 3 demonstrate that although clotrimazole is a strong Pdr5p substrate, it is also a potent inhibitor of ATPase activity. Previous work with ABC transporters demonstrated that some transport substrates inhibit ATPase activity; for example, cyclosporine A is transported by P-gp but inhibits P-gp-mediated ATP hydrolysis (*30*). It is clear from the autoradiogram depicted in Figure 4A that clotrimazole and tritylimidazole do not affect the photo-cross-linking of 8-azido[α-^{32}P]ATP to Pdr5p (compare lanes 9, 11, and 12) under nonhydrolyzable conditions. Therefore, the inhibition of ATP hydrolysis does not appear to be attributable to a direct interaction of clotrimazole with the NBDs but rather to an allosteric interaction at an alternative site. It was also plausible that clotrimazole influences ATP hydrolysis by either increasing or decreasing the affinity for the substrate (ATP) or the product (ADP). Thus, we estimated the apparent affinity of the nucleotide analogues 8-azido[α-^{32}P]ATP and 8-azido-[α-^{32}P]ADP in the absence and presence of 20 μM clotrimazole at 30 °C. Panels B and C of Figure 4 show that clotrimazole has no effect on the concentration-dependent cross-linking of Pdr5p with either 8-azido[α-^{32}P]ATP or 8-azido[α-^{32}P]ADP.

These results are consistent with the finding that a range of clotrimazole concentrations (0−10 μM) did not influence the K_m(ATP) for Pdr5p-mediated ATP hydrolysis, which is ~1.5 mM for all of the curves shown in Figure 4D. Instead,

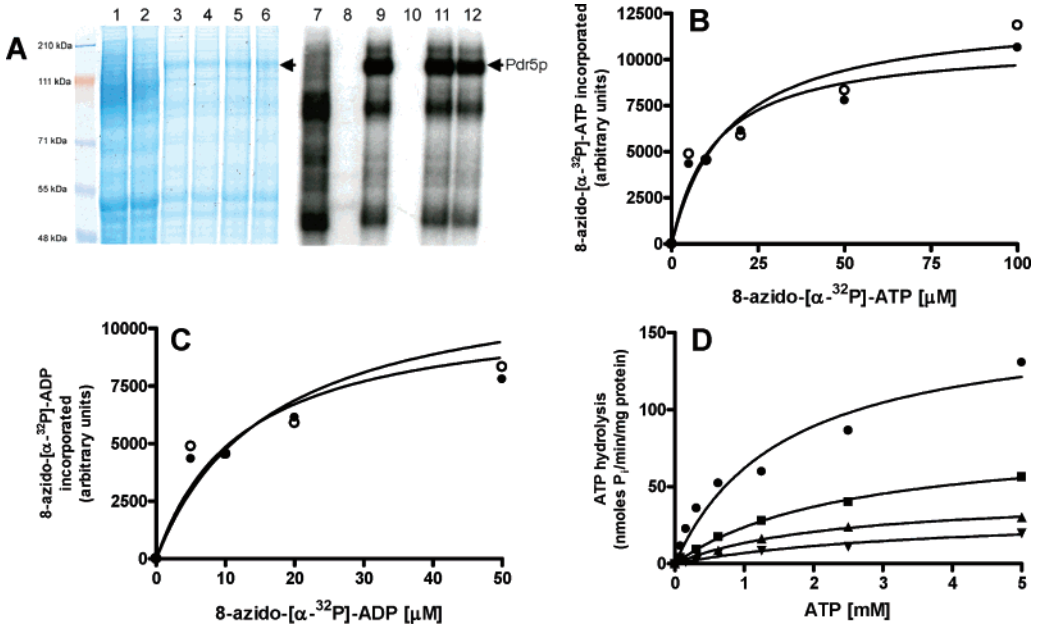

FIGURE 4: Clotrimazole behaves as a noncompetitive inhibitor of Pdr5p-mediated ATP hydrolysis. (A) Photolabeling of Pdr5p with 8-azido-[α-^{32}P]ATP and effect of transport substrates. Purified plasma membranes were prepared as previously described (*13*). In samples using AD124567 (Pdr5p$^+$), 40 μg of membrane protein was used for photolabeling, while 60 μg of protein was used for the AD1-7 (Δ*pdr5*) vesicles to "overload" the control. Following cross-linking, samples were run on a 7% Tris-acetate gel. The gel was stained, dried, and exposed to X-ray film. Lanes 1−6 depict a gel stained with Colloidal Blue and lanes 7−12 the autoradiogram for the same gel. The Pdr5p bands are marked by arrows: lanes 1 and 7, AD1-7 (Δ*pdr5*); lanes 2 and 8, AD1-7 with 2 mM ATP; lanes 3 and 9, AD124567 (*Pdr5*$^+$); lanes 4 and 10, AD124567 with 2 mM ATP; lanes 5 and 11, AD124567 with 5.0 μM clotrimazole; lanes 6 and 12, AD124567 with 5.0 μM tritylimidazole. Similar results were obtained in two additional experiments. (B) Incorporation of 8-azido[α-^{32}P]ATP into Pdr5p at 30 °C in the absence (○) and presence (●) of 20 μM clotrimazole. (C) Incorporation of 8-azido[α-^{32}P]ADP into Pdr5p at 30 °C in the absence (○) and presence (●) of 20 μM clotrimazole. The apparent K_d values were determined by using GraphPad Prism curve-fitting software. (D) Effect of clotrimazole on the kinetic parameters (K_m and V_{max}) of the Pdr5p-mediated ATPase reaction. Pdr5p-mediated ATP hydrolysis was monitored at 35 °C as described in Experimental Procedures in the presence of increasing concentrations of ATP. The experiments were performed in the presence of 0 (●), 5 (■), 7.5 (▲), and 10 μM (▼) clotrimazole. The kinetic parameters were determined by using GraphPad Prism.

Table 2: Inhibition of Pdr5p-Specific NTPase Activities by Vi and BeFx[a]

condition	ATP	UTP	GTP
control	178	28.9	74.4
with 300 μM Vi	12.4 (93%)	9.75 (66%)	41.0 (45%)
with BeF_x (1.25 mM NaF, 0.25 mM $BeSO_4$)	25.7 (86%)	3.25 (89%)	37.4 (50%)

[a] Specific activities are given as nanomoles per minute per milligram of total vesicle protein. Inhibition (%) is shown in parentheses.

increasing concentrations of clotrimazole cause a progressive decline in the V_{max} of the reaction.

The Intracellular Levels of Clotrimazole Used in Transport Studies Should Eliminate ATPase Activity and Thus Preclude Transport. Although clotrimazole and tritylimidazole are potent inhibitors of Pdr5p ATPase activity, transport of clotrimazole proceeds with approximately the same efficacy from at least 0.6 to 15 μM. An obvious explanation for this contradiction is that the effective intracellular concentrations of clotrimazole and tritylimidazole are sufficiently low to preclude ATPase inhibition. We estimated the intracellular concentration from the levels of accumulated [^{3}H]clotrimazole. When 5.0 μM clotrimazole is used in a transport assay, ~12 pmol/10^7 cells is observed in the Pdr5p strain. Assuming the volume of a yeast cell to be 7×10^{-14} L (*31*), the total volume of 10^7 yeast cells is 0.7 μL and the intracellular concentration is 17 μM. This concentration should eliminate all ATPase activity. In fact, when these calculations are performed, it is clear that even the lowest concentration of clotrimazole used in the transport assay (0.6 μM) results in an inhibitory intracellular concentration (~3.5 μM). Furthermore, confluent growth of the Pdr5p strain is observed even on medium containing 15 μM clotrimazole.

A similar conclusion is reached from previous work with tritylimidazole transport (*5*). In this instance, addition of 5.0 μM tritylimidazole led to an intracellular accumulation of 10 pmol/10^7 cells (*5*) or an estimated concentration of ~14 μM in the Pdr5p strain.

A second possibility is that clotrimazole is sequestered in the vacuole, thereby reducing the concentration to a level permitting ATPase activity. At present, the only means of doing this in yeast that has been documented for nonpeptide inhibitors is the glutathione-mediated vacuole sequestering of heavy metal ions and drugs by the ABC transporter Ycf1p (*32*). As this transporter has been deleted from the strains used in this study, another undescribed transport system would need to be invoked.

Pdr5p-Mediated GTP Hydrolysis Is Resistant to Clotrimazole. A more appealing explanation for the disparity between in vivo transport and in vitro ATPase assay results comes from the observation that Pdr5p appears to exhibit a high, specific NTPase activity (*10*). This suggested to us that the NTPase of other nucleotides might be less sensitive to trityl derivatives and thus provide an alternative energy source for Pdr5p-mediated efflux. This would not necessarily be at odds with our observation that 2-deoxyglucose abolished transport of clotrimazole because ATP is required for

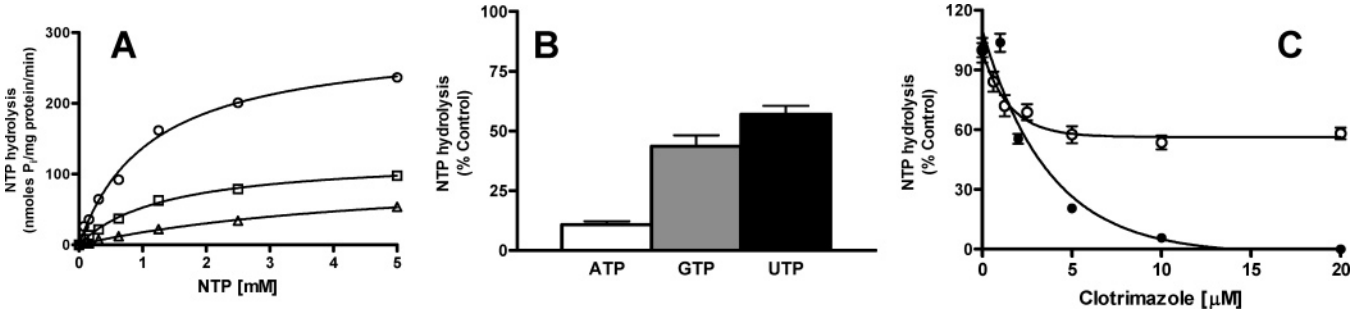

FIGURE 5: Analysis of Pdr5p NTPase activity. NTPase activity was performed at 35 °C as described in Experimental Procedures. (A) Pdr5p-specific NTPase activity was monitored by plotting the difference between the $Pdr5p^+$ and $Pdr5p^-$ vesicles in the presence of increasing concentrations of ATP (○), GTP (□), or UTP (△). The kinetic parameters of Pdr5p-mediated NTPase activities were estimated by using the curve fitting software GraphPad Prism. (B) Effect of 10 μM clotrimazole on NTPase activities using ATP (white bar), UTP (gray bar), and GTP (black bar). The error bars represent the standard error ($n = 3$). (C) ATPase (●) and GTPase (○) activities as a function of clotrimazole concentration. The error bars represent the standard deviation ($n = 3$).

the biosynthesis of other nucleotides under both de novo and salvage pathways. The intracellular concentration of UTP and GTP in yeast cells is reported to be approximately one-third of that of ATP (*33*), which would place these concentrations in the 1.0 mM range. This figure should be regarded as a rough estimate. In any case, concentrations of free ATP and GTP available to power transport in yeast are not known. The reported K_m(UTPase) for Pdr5p is 0.35 mM (*10*), which therefore suggests that this activity could serve a physiological purpose. This value, however, was obtained from Pdr5p reconstituted in liposomes, and this preparation had very low activity.

We determined the kinetic behavior of Pdr5 UTPase and GTPase activity at 35 °C in our membrane vesicle preparations. The activity was initially assayed at 35 °C in the isogenic Pdr5p and Δ*pdr5* strains in the presence and absence of vanadate, Vi, and beryllium fluoride (BeFx). These data are found in Table 2. The basal activity for all three nucleotides is robust compared with those of most other ABC transporters. The ATPase activity is greatest, followed by GTPase (∼70 nmol min^{-1} mg^{-1}) and UTPase (∼35 nmol min^{-1} mg^{-1}) activity. We also observed that the effect of Vi and BeFx was nucleotide-dependent. As expected, the ATPase activity is very sensitive to these compounds. The GTPase activity, however, is much less so. Furthermore, K_m-(ATP) and K_m(GTP) are comparable, 1.9 and 1.36 mM, respectively. However, in contrast to previously reported results, we found a very high K_m(UTP), which may be too large to be physiologically significant (>5 mM). These results (Figure 5A) suggest that it is plausible that GTP could be used as an energy source for transport.

A comparison of the effect of 10 μM clotrimazole on the ATPase, GTPase, and UTPase activity of Pdr5p demonstrates that GTPase and UTPase activities are inhibited far less than ATPase activity (Figure 5B). Thus, although 91% of the ATPase activity was eliminated by treatment with clotrimazole, approximately 50% of the GTPase activity was retained. We also demonstrate by varying the clotrimazole concentration that even at saturating concentrations of clotrimazole, nearly 60% of the GTPase activity is retained, whereas ATPase activity is completely inhibited (Figure 5C).

The relatively low K_m(GTPase) and the significant resistance of this activity to clotrimazole suggest that Pdr5p may use other nucleotides as a backup energy sources when ATPase activity is absent.

The GTPase Activity Is Specific to Pdr5p. The absence of any GTPase activity in the AD1-7 Δ*pdr5* strain argues that the GTPase activity that we observe in its isogenic Pdr5p partner is Pdr5p-specific. However, as the two strains have been separated for more than 8 years, it is not unreasonable to posit that the AD1-7 strain lost an additional NTPase activity unrelated to Pdr5p. The relatively high resistance of the GTPase activity to BeFx and Vi also suggests that this is plausible. To address this possibility, we carried out the experiment depicted as a schematic in Figure 6A. Transformation of AD1-7 with pSS607 created the JG2000 strain which has Pdr5p ATPase activity as already discussed above (see Figure 1B). This strain contains the original Δ*pdr5* mutation and a wild-type copy of the gene separated by plasmid sequences that also contain the *URA3* selectable marker. As a result of relatively rare, spontaneous homologous recombination, loss of one copy of *PDR5* (mutant or wild type each with 50% probability) and the intervening plasmid sequences including the *URA3* gene occurs at low frequency. Such colonies can be recovered by negative selection on 5FOA medium. These $ura3^-$ segregants will therefore have a single copy of the mutant (cycloheximide-hypersensitive) or wild-type (cycloheximide-hyperresistant) gene. In this study, 12 of 22 colonies were sensitive to cycloheximide which is close to the expected frequency of 50%. One such sensitive segregant (JG2002) was compared to AD1-7 and JG2000 (AD1-7 with pSS607) on medium containing 0.3 μM clotrimazole. As shown in Figure 6B, although Pdr5p-bearing strain JG2000 shows characteristic resistance to clotrimazole, the AD1-7 and JG2002 strains are phenotypically indistinguishable and drug-sensitive. The transport capabilities of JG2000 and JG2002 are shown in Figure 6C. The latter has lost the ability to efflux clotrimazole and retains twice the amount of compound when compared to the pSS607 (*PDR5*)-bearing strain. We also show that vesicles prepared from the $Pdr5p^+$ JG2000 cells exhibit ATPase and GTPase activities (Figure 6D). In addition, consistent with previous results (Figure 5B), the ATPase activity is far more sensitive to 10 μM clotrimazole (84%) than the GTPase activity (18%). Finally, we demonstrate that the GTPase present in JG2000 is eliminated in JG2002 which has been cured of *PDR5* and retains only the mutant copy of the gene (Figure 6E).

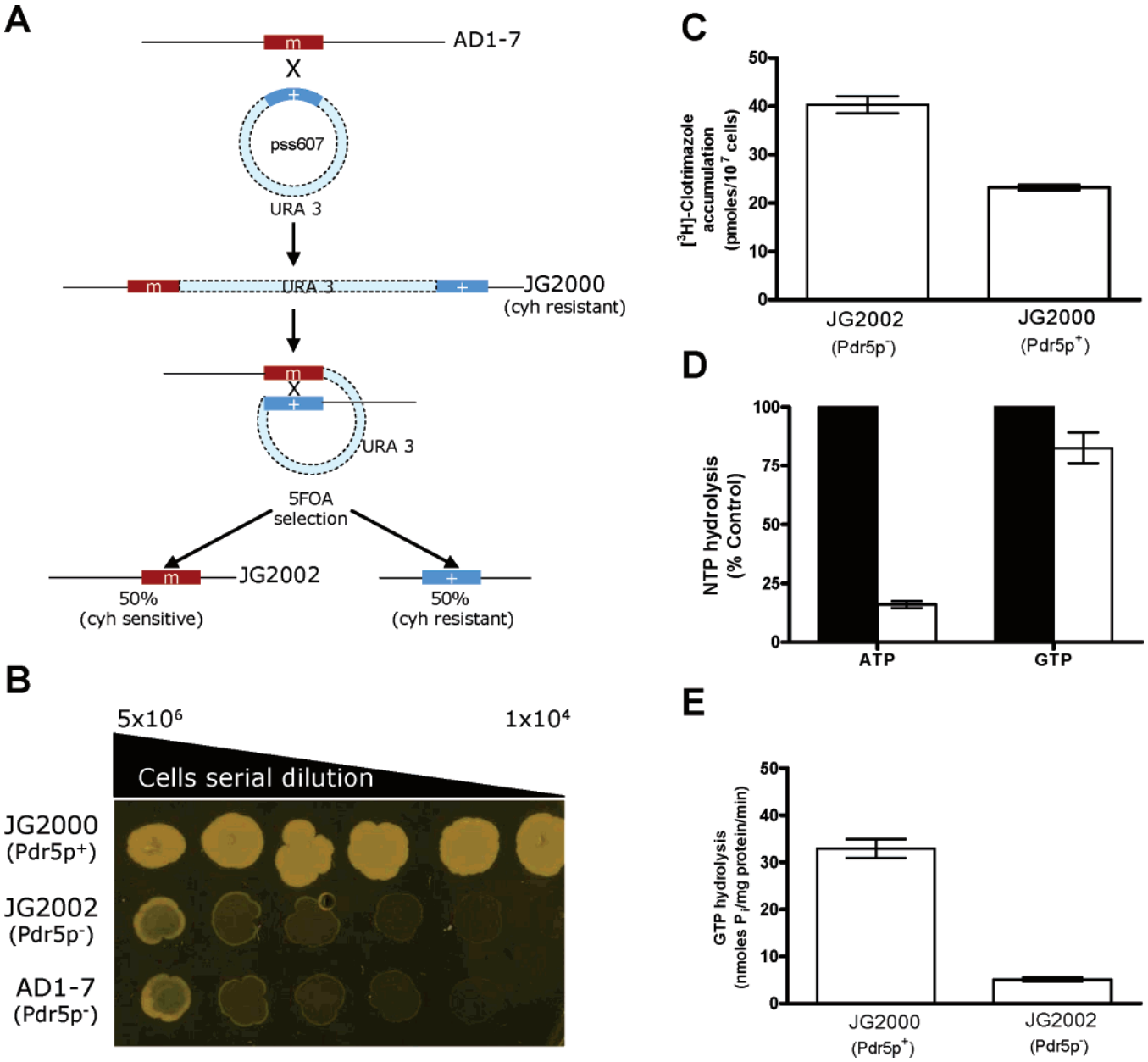

FIGURE 6: Specificity of Pdr5p GTPase activity. (A) The schematic shows the construction of JG2000 and JG2002. Δ*pdr5* strain AD1-7 was transformed with pSS607 to create JG2000. This strain contains two copies of *PDR5:* a mutant one designated as m that is missing 1200 bp including the promoter and the beginning of the downstream region and the wild-type copy (+) separated by plasmid DNA (dashed lines) containing a *URA3* gene. Spontaneous homologous recombinants containing one copy of the gene are obtained by selection on 5FOA medium. Colonies containing the m copy are sensitive to cycloheximide and make up, as expected, ~50% of the segregants (10 of 22). The AD1-7, JG2000, and JG2002 strains are used in subsequent experiments. (B) Spot tests of strains were carried out on medium containing 0.3 μM clotrimazole. Cultures of the three strains were grown to saturation. The cell concentration was determined, and serial dilutions were performed so that the concentration of cells in 5 μL ranged from 5×10^6 (left) to 10^4 (right). The plates were photographed at 72 h. (C) Transport of 5 μM [^{3}H]clotrimazole in JG2000 (Pdr5p$^+$) and JG2002 (Pdr5p$^-$) assessed as described in the legend of Figure 3. (D) Vi-sensitive ATPase and GTPase activity was determined in the absence (black bars) or presence (white bars) of 10 μM clotrimazole using vesicles prepared from JG2000 (Pdr5p$^+$) cells. (E) Vi-sensitive GTPase activity was determined in vesicles prepared from JG2000 and JG2002 (JG2000 cured of plasmid on 5FOA medium) cells. Error bars represent the standard deviation ($n = 3$).

Pdr5p-Specific, GTP-Driven Uptake of [^{3}H]Chloramphenicol in Plasma Membrane Vesicles. The results thus far indicate that Pdr5p could use GTP as an energy source for the transport of xenobiotic substrates. To determine if this was indeed the case, we tested the ability of GTP to promote uptake of [^{3}H]chloramphenicol in plasma membrane vesicles. Unfortunately, it was not possible to do this with [^{3}H]-clotrimazole as this substrate binds tenaciously to the membrane filters used in our uptake assays, and all attempts to preblock the filters failed. Therefore, we took advantage of previous work demonstrating that [^{3}H]chloramphenicol is transported specifically by Pdr5p (*5*, *34*). Furthermore, null alleles of *PDR5* are 20−40 times more sensitive to chloramphenicol than their isogenic *PDR5* counterparts. To ensure specificity, we carried out these experiments with the JG2000 and JG2002 strains described in Figure 6. The vesicle uptake studies clearly demonstrate that in the presence of 5 mM GTP, there is a time-dependent, ~3-fold increase in the extent of chloramphenicol uptake compared to that in vesicles lacking Pdr5p. Also, there was no accumulation of chloram-

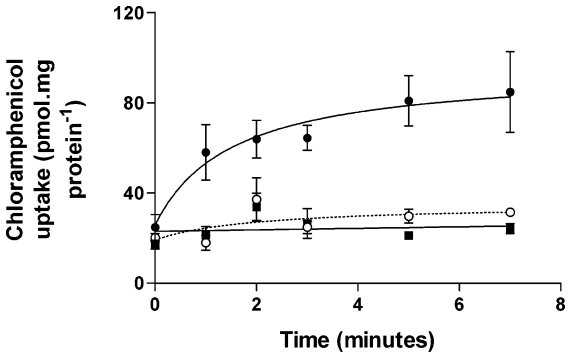

FIGURE 7: Time-dependent uptake of [^{3}H]chloramphenicol into inside-out plasma membrane vesicles. Purified plasma membrane vesicles were prepared as described previously (*13*) except that following sucrose gradient centrifugation vesicles were diluted and resuspended in transport buffer [10 mM Hepes (pH 7.5), 20 mM KCl, 250 mM sucrose, and 10 mM $MgCl_2$]. Uptake of [^{3}H]Chl was monitored over time in vesicles from JG2000, Pdr5p$^+$ (●) and JG2002, Pdr5p- (■) cells as described in Experimental Procedures in the presence or absence of 5 mM GTP. Uptake of chloramphenicol in the Pdr5p$^+$ vesicles was also monitored in the absence of nucleotide (○). The data points represent the mean, and the error bars represent the standard deviation ($n = 4$).

phenicol in the absence of nucleotide even in the Pdr5p$^+$ vesicles. As the proportion of vesicles in our assay that are inside-out is not known, we are probably underestimating the degree of transport. The difference between the *PDR5* and Δ*pdr5* strains that we observe in these in vitro experiments is remarkably like the difference (3-fold) observed in whole cell transport studies (*5*).

DISCUSSION

Pdr5p is a major yeast multidrug transporter that has been the subject of considerable study. Surprisingly, its ATPase activity has not yet been extensively characterized. Save for an important, initial report (*10*), no further enzymatic studies have been carried out, even though several substrate-specific mutants are known (*35*, *36*). In this study, we characterize ATP hydrolysis in plasma membranes prepared from yeast strain AD124567. This strain overexpresses Pdr5p but is otherwise isogenic to yeast strain AD1-7, which lacks all major plasma membrane ABC transporters. We demonstrate that the Pdr5p-mediated ATP hydrolysis is sensitive to the transition state inhibitor, Vi. The Vi-sensitive ATP hydrolysis at 35 °C shows Henri−Michaelis−Menten kinetics with a K_m(ATP) of ∼1.9 mM and a V_{max} of 85.6 nmol of P_i (mg of protein)$^{-1}$ min^{-1} (Figure 1C). In general, ABC proteins, including the recently characterized Cdr1p, exhibit a low affinity for ATP in the range of 0.3−2.0 mM (*3*, *12*), and the value for Pdr5 is consistent with these reports. We also show, as have others (*23*), that the photoaffinity nucleotide analogue, 8-azido[α-^{32}P]ATP, binds specifically to the NBD(s) of Pdr5p, as this binding can be reversed by addition of ATP (Figure 4).

Save perhaps for its high basal ATPase activity, the behavior of Pdr5p does not seem particularly unusual for an ABC transporter. The contradictory effect of clotrimazole and tritylimidazole in ATPase and transport assays, however, is striking and suggests that this transporter might use more than one kind of nucleotide as an energy source for transport. A previous study reported significant Pdr5p NTPase activity (*10*). In this report, we demonstrate that (1) Pdr5p GTPase activity (but probably not UTPase activity) exhibits a K_m consistent with a physiological role, (2) the GTPase and UTPase activities are considerably more resistant to inhibition by clotrimazole than is the ATPase activity, and (3) GTP supports the Pdr5p-specific uptake of [^{3}H]chloramphenicol in plasma membrane vesicles. Although chloramphenicol does not inhibit Pdr5p ATPase activity (data not given), this substrate shares an in vivo transport site with clotrimazole (*5*).

Our observations are intriguing for several reasons. It is likely that the ability of Pdr5p to use different nucleotides to fuel transport gives Pdr5p greater substrate specificity. It is also possible that this could be a feature of transporters that are found in relatively small genomes such as *Saccharomyces*. In this regard, it is noteworthy that the Ycf1 transporter located in the vacuolar membrane can also use GTP (*37*). It is plausible that in these organisms, relatively few drug pumps must eliminate many compounds; presumably more complex cells employ a greater division of labor. Furthermore, Pdr5p does not contain some of the characteristic, well-conserved NBD sequence motifs. The Walker A, the extended Walker B, and the H-loop are degenerated in NBD1, and the C-loop of NBD2 deviates from the canonical sequence. It is plausible that the novel behavior we observe is not found in most conventional mammalian drug transporters due to these variations in the NBD sequences. The Pdr5p sequences do, however, bear a striking resemblance to several known pathogenic fungal efflux pumps, notably Cdr1p of *C. albicans* and CnAfr1p of *Cryptococcus neoformans* (*38*). Significant NTPase activity has also been observed with the *C. albicans* Cdr1p transporter, although it is not clear whether the activity is physiological (*21*). It would be interesting to determine whether these pathogenic fungal proteins exhibit NTPase behavior analogous to that of Pdr5p.

NTPase activity is also observed with some mammalian transporters, such as the ABCG5−ABCG8 heterodimer, and it has been suggested that GTP might be used to power transport (*39*). These results underscore the point that, at least in yeast, modulators probably cannot be targeted against one specific NTPase activity, although drugs that block the binding of nucleotide to NBDs may be beneficial. Such a mechanism has been demonstrated for disulfiram in mammalian cells and in *C. albicans* (*40*, *41*).

Although clotrimazole exhibits complete, concentration-dependent inhibition of ATPase activity, it is a noncompetitive inhibitor acting at a location that is distinct from (1) the NBDs and (2) the drug-substrate transport sites. The first is supported by the fact that these compounds affect the V_{max} but not the K_m(ATP). Furthermore, the cross-linking of 8-azido[α-^{32}P]ATP is not affected by the presence of clotrimazole, whereas the cross-linking is completely inhibited by excess ATP. The data presented as Supporting Information address the second issue. 3,9-Diacetylcarbazole inhibits the transport of [^{3}H]clotrimazole, but tritylimidazole has no effect, suggesting that 3,9-diacetylcarbazole and clotrimazole share a transport-substrate site which is distinct from the site of tritylimidazole transport. Data presented in Table S1 show that 3,9-diacetylcarbazole has no effect on Pdr5p-mediated ATP hydrolysis (Table S1). Thus, if the inhibition of ATP hydrolysis occurs due to interaction(s) of clotimazole at the transport-substrate site, addition of excess

3,9-diacetylcarbazole should protect against inhibition. The observation that an excess of 3,9-diacetylcarbazole does not reverse the clotrimazole-induced inhibition of ATP hydrolysis (Table S1) suggests that the site of inhibition of Pdr5p-mediated ATP hydrolysis is distinct from the transport-substrate site. Models that invoke a clotrimazole-stimulated posthydrolysis conformation that favors increased ADP affinity at the expense of further ATPase activity are also improbable because such a mechanism would produce a strengthened signal in the presence of clotrimazole in cross-linking experiments with either azidoATP or azidoADP. Therefore, it would appear that clotrimazole allows normal binding of nucleotide but prevents a conformation necessary for hydrolysis. It is likely that the type of allosteric interaction between clotrimazole and Pdr5p is quite common, because it has already been documented well with mammalian P-gp (*42*, *43*). The ability of Pdr5p to use nucleotides other than ATP is a way to relieve this inhibition and thus effectively increase the size of its repertoire of substrates.

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SUPPORTING INFORMATION AVAILABLE

Figure S1 and Table S1 demonstrate that although 3,9-diacetylcarbazole and clotrimazole share a transport site, the former is unable to reverse inhibition of ATPase by the latter. This material is available free of charge via the Internet at http://pubs.acs.org.

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