Characterization of Cdr1p, A Major Multidrug Efflux Protein of *Candida albicans:* Purified Protein Is Amenable to Intrinsic Fluorescence Analysis†

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Received September 20, 2005; Revised Manuscript Received December 22, 2005

ABSTRACT: *Candida* drug resistance protein 1 (Cdr1p), an ATP-dependent drug efflux pump, confers multidrug resistance in immunocompromised and debilitated patients. A member of the ATP-binding cassette (ABC) superfamily of membrane transporters, Cdr1p contains two nucleotide binding/utilization sites (NBDs) and two transmembrane domains (TMDs). We had earlier characterized Cdr1p by its overexpression as a GFP-tagged fusion protein that elicits oligomycin-sensitive ATPase activity and is linked to drug extrusion. However, it is essential to have highly purified Cdr1p to understand the detailed molecular basis of structure and functions of this protein. In this study, we have developed a two-step purification protocol using stably overexpressed His-tagged Cdr1p in *Saccharomyces cerevisiae.* Purified Cdr1p exhibited divalent cation-dependent ATPase activity [$\sim$1.2 μmol (mg of protein)$^{-1}$ min^{-1}] with an apparent K_M in the range of 1.8 to 2.1 mM and V_{max} between 1.0 and 1.4 μmol (mg of protein)$^{-1}$ min^{-1}. Unlike its close homologue human P-gp/MDR1, purified Cdr1p only moderately displayed drug stimulated ATPase activity. By exploiting intrinsic fluorescence intensity of purified Cdr1p, which contains 24 tryptophan residues, we could monitor defined conformational changes upon substrate drug and ATP binding. It is observed that ATP binding to Cdr1p (K_d = $\sim$1.7 mM) is not a prerequisite for drug binding, and both the mechanisms of drug as well as ATP binding, which induce specific conformational changes, occur independent of each other. Our study for the first time provides a catalytically active purified ABC transporter from a fungal pathogen, which is amenable to fluorescence measurements and thus would be useful in understanding the molecular basis of antifungal transport.

One of the most clinically significant mechanisms of azole resistance in pathogenic yeast *Candida albicans* is the overexpression of the multidrug transporter protein *Candida* drug resistance protein 1 (Cdr1p), belonging to the ATP binding cassette (ABC)[1] superfamily of transporters (*1*−*3*). This is well evidenced in reports from fluconazole-resistant clinical isolates of *C. albicans*, where enhanced expression of Cdr1p has been shown to help the pathogen to efflux therapeutic azoles and facilitate its survival (*4*−*6*). Thus, Cdr1p has not only acquired significant clinical importance but is also considered an important target in designing any strategy to combat antifungal resistance (*2*, *7*).

The *CDR1* gene encodes an integral plasma membrane protein of 1501 amino acids with a predicted molecular mass of 169.9 kDa (*8*). On the basis of its amino acid sequence, Cdr1p is predicted to consist of two homologous halves, each comprising one N-terminal hydrophilic nucleotide-binding domain (NBD) followed by a C-terminal hydrophobic transmembrane domain (TMD). Each TMD comprises six transmembrane segments (TMS), which are envisaged to confer substrate specificity to Cdr1p. The substrates of Cdr1p vary enormously and include structurally unrelated compounds such as azoles, lipids, and steroids (*2*, *7*). This promiscuity toward substrates is a characteristic feature of most ABC-type drug transporters and hence makes their functionality all the more complex to understand (*9*). Another feature of these ABC-type transporters including Cdr1p is that they utilize energy released from nucleotide hydrolysis to power the transport of substrates across the plasma membrane.

In an effort to develop an understanding of the molecular details of drug binding and transport by Cdr1p, we have earlier observed that several amino acid residues of TMS6, TMS11, and TMS12 are critical for its proper assembly in plasma membrane and as well as for drug transport (*10*−*13*). Recent basic structural and functional analysis of Cdr1p has suggested that the drug binding sites are scattered throughout the protein, and probably more than one residue

† The work presented in this paper has been supported in parts by grants from Department of Biotechnology, (DBT/PR4862/BRB/10/360/2004), Council of Scientific and Industrial Research (37(1132)/03/EMR-II), Department of Science and Technology (SP/SO/D01/2001), and European Commission, Brussels (QLK-CT2001-02377). S.S. and V.R. acknowledge the University Grants Commission, India, for the support in the form of Research Fellowships. D.B. is the recipient of a Senior Research Fellowship from the Council for Scientific and Industrial Research (CSIR), India.

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[1] Abbreviations: ABC, ATP binding cassette; Aniso, anisomycin; CFTR, cystic fibrosis transmembrane conductance regulator; CM, crude membrane; Cyclo, cycloheximide; DDM, *n*-dodecyl β-D-maltoside; EDTA, ethylenediaminetetraacetic acid; Flu, fluconazole; IMAC, immobilized metal affinity chromatography; Mic, miconazole; MS, mass spectroscopy; MALDI, matrix assisted laser desorption ionization; NBD, nucleotide binding domain; PM, plasma membrane; TNP-ATP, 2′(3′)-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate; TMD, transmembrane domain; TMS, transmembrane segment.

10.1021/bi0519147 CCC: $33.50
Published on Web 01/31/2006

Table 1: List of Strains Used

strain	description	ref
AD1−8u⁻	*MATα pdr1−3 his1 ura3 Δyor1::hisG Δsnq2::hisG Δpdr5::hisG Δpdr10::hisG Δpdr11::hisG Δycf1::hisG Δpdr3::hisG Δpdr15::hisG*	*20*
AD1002	*MATα pdr1−3 his1 ura3Δyor1::hisG Δsnq2::hisG Δpdr5::PDR5PROM-CDR1−CDR1STOP Δpdr10::hisG Δpdr11::hisG Δycf1::hisG Δpdr3::hisG Δpdr15::hisG*	*20*
VyCDR1H	AD1−8u⁻ cells harboring CDR1$(His)_6$ ORF integrated at PDR5 locus	this study
SSCH−C193A	CDR1-$(His)_6$ cells carrying C193A mutation in CDR1 ORF and integrated at PDR5 locus	this study
SSCH−W326A	CDR1-$(His)_6$ cells carrying W326A mutation in CDR1 ORF and integrated at PDR5 locus	this study

Table 2: List of Oligonucleotides Used

oligonucleotide	sequence	purpose
CDR1F (1210)	5′ GCTACACTAGTATGTCAGATTCTAAGATGTCGTCG 3′	forward primer to amplify CDR1 ORF having SpeI
HR2	5′CTGACACTAGTTTAGTGATGGTGATGATGGTGA TGCGCTGCTTTCTTATTTTTTTTCTCTCTGTTAC 3′	reverse primer to introduce six histidines at C-terminal having Spe1site
CDF-15	5′ GCCAATTTGGCTTTTTTGTTATTTACTATG 3′	primer to check correct clone
CDR1R	5′ TTGACTAATTGCCTTTTCTA 3	primer to check right orientation
CDF-18	5′ CAATACTTTCTTGAAATCAATTAATTCATTATACAG 3′	primer to check introduction of six histidines
C193A/F	5′ GGGAGACCCGGTGCTGGTGCATCCACATTGTTAAAGACC 3′	forward primer to mutate Cys193 to Ala
C193A/R	5′ GGTCTTTAACAATGTGGATGCACCAGCACCGGGTCTCCC 3′	reverse primer to mutate Cys193 to Ala
W326A/F	5′ GGTGCTAATATCCAATGTGCCGATAATGCCACTAGAGGG 3′	forward primer to mutate Trp326 to Ala
W326A/R	5′ CCCTCTAGTGGCATTATCGGCACATTGGATATTAGCACC 3′	reverse primer to mutate Trp326 to Ala

in different transmembrane helices are involved in drug binding and extrusion (*11*−*13*). Sufficient information is still lacking to predict where the most common antifungals such as fluconazole, itraconazole, and ketoconazole bind and get expelled. Despite limited success with purified domain and with plasma membrane of Cdr1p overexpressing cells (*11*−*16*), there is a greater need to have highly purified and active Cdr1p for detailed structural and functional studies. Unlike mammalian ABC transporters, there are very limited studies in which fungal ABC purified proteins have been subjected to structural and functional studies (*17*, *18*). In contrast, human MDR1/P-gp and MRPs of mammalian origin have been purified and as a result helped in understanding the drug transport cycle and revealed structural details of this class of proteins.

In this study, we have developed a two-step purification protocol for Cdr1p using immobilized metal affinity chromatography (IMAC) and anion exchange chromatography. The purified Cdr1p exhibits oligomycin-sensitive ATPase activity, which is only moderately activated with selected drug substrates. The purified protein, which possesses 24 Trp residues, is readily amenable to fluorescence spectroscopic analysis. We show that ATP binding to Cdr1p is not a prerequisite for drug binding and both drug as well as ATP binding, which induce specific conformational changes, could occur independent of each other.

EXPERIMENTAL PROCEDURES

Materials. *n*-Dodecyl-*β*-D-maltoside, imidazole, lysolecithin, Q-Sepharose Resin, TNP-ATP, ribonucleotides (ATP, CTP, GTP, UTP), anisomycin, cycloheximide, miconazole, fluphenazine, oligomycin, and other molecular biology grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Ni^{2+}-NTA Superflow and Anti Pentahis monoclonal antibody were from Qiagen. Sequencing kit (Sequenese Version 2.0 Enzyme), protease inhibitors and ECL kit for Western blotting were obtained from Amersham Biosciences Ltd. The Pfu DNA polymerase for PCR amplification was purchased from Stratagene Inc (La Jolla, CA). Oligonucleotides used in this study were commercially procured from Sigma. Fluconazole was kindly provided by Ranbaxy Laboratories (New Delhi, India). Polyclonal antibody to Cdr1p against the peptide CQSNKISKKKEKDDYVDY (amino acids 965−979, part of the putative C-terminal NBD) which represented the most antigenic epitope of Cdr1p was commercially synthesized and purchased from Covance Research Products, Inc.

Methods. Bacterial and Yeast Strains and Growth Media. Plasmids were maintained in *Escherchia coli* XL-1 blue. *E. coli* was cultured in Luria-Bertini medium (Difco, BD Biosciences) to which ampicillin was added (100 μg/mL). The *S. cerevisiae* strains used were AD1−8u⁻ and AD1002 (provided by Richard D.Canon, University of Otago, and Dunedin, New Zealand). VyCDR1H, SSCH−C193A and SSCH−W326A were AD1−8u⁻ derivatives expressing Cdr1p-$(His)_6$ and its mutant proteins (Table 1). The yeast strains were cultured in yeast extract-peptone-dextrose (YEPD) broth (Difco, BD Biosciences). For agar plates, 2.5% (w/v) Bacto agar (Difco, BD Biosciences) was added to the medium.

Molecular Cloning. Plasmid pS12−35 was used to amplify *CDR1*−ORF (*8*). The primers (Table 2) used for this purpose allowed the introduction of Spe I restriction sites at the 5′ and 3′ termini, including six consecutives histidines at 3′ end of the amplicon. The resultant amplicon was digested with Spe I and ligated at the corresponding sites of the linearized pSK−PDR5PPUS vector. The resultant construct psCdr1H was sequenced to confirm positive clone in the right orientation and six histidines were introduced at the 3′ end.

Site-Specific Mutagenesis and Development of Transformants. Site-directed mutagenesis was performed by using the Quick Change mutagenesis system from Strategene (La Jolla, CA). The mutations were introduced into plasmid psCdr1H according to the manufacturer's instructions using primers mentioned in Table 2, and the desired nucleotide sequence alterations were confirmed by DNA sequencing

of the ORF. The wild-type construct psCdr1H and its mutated versions were linearized with Xba I and used to transform AD1−8u⁻ cells for uracil prototrophy by the lithium acetate transformation protocol (*19*).

Genomic DNA Extraction and Southern Analysis of CDR1 ORF in S. cerevisiae. Genomic DNA was isolated from *S. cerevisiae* cells as described previously (*11*). Southern analysis was performed to check for single copy integration into the genomic DNA of AD1−8u⁻ cells according to the protocol discussed previously (*20*).

Isolation and Solubilization of Crude Membrane (CM). Crude membranes (CM) were prepared from Cdr1p-$(His)_6$ expressing *S. cerevisiae* cells grown in YEPD to late exponential phase. The cells were broken with glass beads by vortexing the cells four times for 30 s each with intermittent cooling on ice. The homogenization medium (buffer A) contained 50 mM Tris (pH 7.5), 2 mM $MgCl_2$ and protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1 μg/mL of each leupeptin, aprotinin and pepstatin). The CM was recovered by centrifugation at 1000*g* to remove unbroken cells and finally pelleting the CM by ultracentrifugation at 1,000,000*g* for 1 h. The CM was resuspended in resuspension buffer (buffer B) having 20 mM Tris (pH 7.5), 2 mM $MgCl_2$, 150 mM NaCl, 20% glycerol, 20 mM imidazole, and the protease inhibitor cocktail in the same concentrations as mentioned in buffer A. The crude membrane was then solubilized using the nonionic detergent *n*-dodecyl β-D-maltoside (DDM) by using protein/detergent (w/w) ratio 1:1. The mixture was allowed to stir previously at room temperature for 5 min and then at 4 °C for 30 min. After being stirred for 30 min, the mixture was spun at 18 000 rpm at 4 °C for 45 min, and soup was collected avoiding the lipids and pelleted materials for further purification of Cdr1p.

Protein Purification. Solubilized crude membranes were added to preequilibrated Ni−NTA Superflow Resin (Qiagen) in 0.05% DDM, 20 mM Tris (pH 7.5) and kept for shaking on Roto-Torque (Cole-Parmer, USA) at 4 °C for 2 h. The resin, bound with protein, was washed extensively with buffer C having 20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 0.1% DDM, 50 mM imidazole, and protease inhibitors in the same concentrations as described previously. The protein was eluted from resin with buffer D containing 50 mM Tris (pH 7.5), 2 mM $MgCl_2$, 10% glycerol, 150 mM NaCl, 500 mM imidazole, 0.05% DDM. The eluted fraction was subjected to extensive dialysis for 12 h with 1 L of buffer having 50 mM Tris (pH 7.5), 2 mM $MgCl_2$, 10% glycerol, 30 mM NaCl, and 0.01% DDM. After dialysis, the protein sample was allowed to bind with Q-Sepharose Resin (Sigma) at 4 °C for 1 h, which was preequilibrated in 50 mM Tris (pH 7.5). The resin bound with protein was then subjected to stringent washing with buffer E [Tris 50 mM (pH 7.5), 2 mM $MgCl_2$, 0.05% DDM and 10% glycerol] having increasing concentrations of NaCl ranging from 100 to 300 mM. Finally, the protein was eluted with buffer F (buffer E + 500 mM NaCl) and diluted with equal volume of buffer E to reduce the NaCl concentration to half. The protein was quantitated according to Bradford (*21*) and BCA (bicinconic acid) kit (Sigma).

Lipid Supplementation of Purified Protein. Purified Cdr1p was first kept for shaking with 0.01% (w/v) of lysolecithin at 4 °C for 1 h and then the protein solution was incubated with SM2 Bio-Beads (Bio-Rad) (5 mg per 100 μL) in the presence of different lipid combinations for 1 h at 4 °C. Protein lipid solution was sonicated thrice at 4 °C in a water bath sonicator (Julabo, Germany) for 30 s, with intervals of 30 s. Cdr1p did not display any specificity to any particular combination; therefore, lipids extracted from host cells AD1−8u⁻ were routinely used in the ratio (lipid/protein) of 1. Finally, protein solution was aliquoted and frozen at −80 °C, which remained active for 4−6 weeks.

Immunodetection and MS Analysis of Cdr1p. Immunodetection of Cdr1p in CM, solubilized fraction, and purified fraction was done with anti-penta His monoclonal (1:5000) and anti-Cdr1p polyclonal antibodies (1:1000) according to the manufacturer's protocol (ECL kit; Amersham Biosciences). For matrix assisted laser desorption ionization (MALDI) MS, the Coomassie blue stained band was excised from the SDS−PAGE gel and subjected to trypsin digestion followed by high mass accuracy MALDI analysis.

ATPase Assay. The end point Pi release colorimetry assay measured ATPase activity of purified Cdr1p as described previously (*20*). Briefly, a 100 μL reaction mixture containing the purified protein was incubated in ATPase buffer (60 mM Tris (pH 7.5) and 8 mM $MgCl_2$) supplemented with 5 mM ATP. The transfer of the mixture from ice to water bath at 30 °C initiated the reaction. The reaction was stopped by the addition of 1 mL of stop solution (0.5% SDS, 2% H_2SO_4, 0.5% ammonium molybdate), followed by the addition of 10 μL of freshly prepared coloring reagent (10% ascorbic acid), and the absorbance was monitored at 750 nm using a UV-2000 Shimadzu spectrophotometer. In the control set of reactions, the purified protein was added after termination of the reaction. To monitor the effect of drugs on ATPase activity, purified Cdr1p in different reaction mixtures was preincubated at 30 °C for 10 min before addition of ATP. The ATPase activity of CM preparation could also be attributed to vacuolar, mitochondrial, and PM-ATPases. Therefore, as a routine, Cdr1p associated ATPase activity of the CM and solubilized fractions was measured as an oligomycin-sensitive release of inorganic phosphate (*11*). We had earlier shown that Cdr1p activity is sensitive to oligomycin (*11*). Percentage increase in oligomycin-sensitive ATPase activity indicated enhancement in Cdr1p associated activity at various stages of purification (discussed below).

Fluorescence Spectroscopic Measurements for Ligand Binding. Experiments were performed at room temperature in a 1-cm path length cuvette using a Cary Eclipse Varian spectrofluorimeter with a slit bandwidth of 5 nm for excitation as well as emission. All spectra were corrected for buffer fluorescence containing lipids and for inner filter effects wherever required. Unless otherwise specified, 1 mL protein samples (25 nM) in 60 mM Tris-HCl pH 7.5 containing 8 mM $MgCl_2$ were used for the intrinsic fluorescence experiments. The protein was stable for the duration of the experiments (approximately 30−45 min) as evidenced by stable fluorescence emission spectra of control samples. Intrinsic Trp fluorescence was measured by excitation at 295 nm and emission spectra recorded between 310 and 400 nm.

Extrinsic fluorescence using TNP-ATP was monitored by excitation at 408 nm and recording emission between 500 and 600 nm. The spectra were corrected for buffer contribution and dilution (<10%). Data obtained were corrected for

inner filter effects using the equation described previously (*16*).

Nucleotide and drug binding was monitored by enhancement of intrinsic tryptophan fluorescence at 350 nm, and the dissociation constants (K_d) were calculated by fitting the data into a Scatchard Plot taking one binding site per molecule, whereas TNP−ATP binding was monitored by enhancement of extrinsic fluorescence of TNP−ATP at 545 nm upon binding to protein and points were fitted into a modified Stern Volmer Plot to obtain dissociation constants.

RESULTS

Expression and Purification of Cdr1p. We have exploited the expression system developed by Nakamura et.al., wherein Cdr1p is stably overexpressed from a genomic *PDR5* locus in a *S. cerevisiae* mutant AD1−8u⁻, which was derived from a *pdr1−3* mutant strain with a gain-of-function mutation in the transcription factor pdr1p, resulting in a constitutive hyperinduction of the *PDR5* promoter (*20*). The high-level expression of Cdr1p was obtained by integration of the *CDR1* ORF to the *PDR5* promoter in AD1−8u⁻, and the resulting strain was designated as AD1002 (*20*). For purification, we introduced six histidines at the C-terminal end of Cdr1p and ensured its single copy integration by Southern analysis (data not shown). The resulting strain overexpressing His-tagged Cdr1p (Cdr1p-$(His)_6$) was designated as VyCdr1H. We ensured that the introduction of six histidines at the C-terminal of Cdr1p does not lead to improper surface localization and impaired functional activity. Western blot of the crude membranes of cells expressing Cdr1p-$(His)_6$ and the host AD1−8u⁻ with anti-penta His monoclonal and anti-Cdr1p polyclonal antibodies confirmed proper membrane localization and expression of the Cdr1p-$(His)_6$ (Figure 1C). Table 3 depicts a drug susceptibility profile of AD1002 (expressing Cdr1p) and VyCdr1H (expressing Cdr1p-$(His)_6$) cells, which confirmed that both constructs display levels of resistance similar to the tested drugs. The specific ATPase activities of plasma membrane fractions from VyCdr1H and AD1002 cells were also comparable (data not shown). Taken together, it was established that histidine tagged Cdr1p was fully functional and properly localized onto the plasma membrane.

The crude membrane isolated from VyCdr1H cells expressing Cdr1p-$(His)_6$ was solubilized by using the detergent *n*-dodecyl β-D-maltoside (protein/detergent ratio 1). Of note, this concentration of detergent was optimal in solublizing Cdr1p, as it did not have any deleterious effect on its activity. The ATPase activity of solubilized fraction was found to be in the range of 70−100 nmol of Pi released (mg of protein)$^{-1}$ min^{-1}. The solubilized fraction was allowed to bind with the Ni−NTA Superflow Resin (Qiagen), and after proper washing with buffer C, the bound protein was then eluted with buffer D (step 1). The eluted fraction was then allowed to bind with an anion exchanger Q-Sepharose Resin (Sigma) after ensuring removal of all the imidazole. The bound protein was subjected to stringent washing with buffer E with increasing concentrations of NaCl (up to 300 mM) and was finally eluted with buffer F containing 500 mM of NaCl (Step 2). As shown in Figure 1A, these two steps of purification yielded Cdr1p that was more than 95% pure (using Quantity One Software; BioRad) and gave a single band on Western blotting with anti-His monoclonal antibody and anti-Cdr1p polyclonal antibody (Figure 1C). The identity of the purified Cdr1p was also verified by MS analysis of tryptic peptides (data not shown).

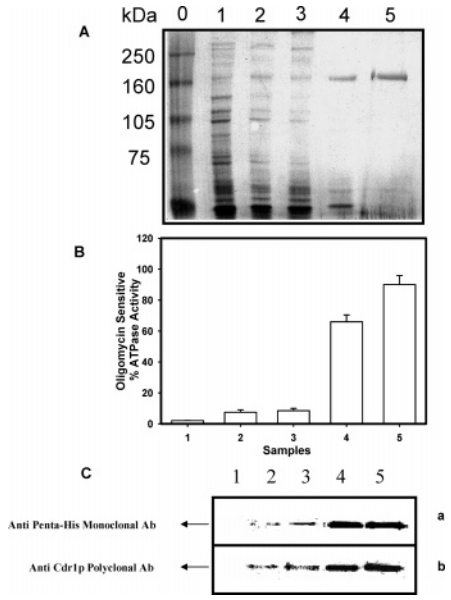

FIGURE 1: Purification and ATPase activity of purified Cdr1p. (A) A silver-stained 8% SDS−PAGE is shown. The lanes are as follows: lane 0, protein molecular mass marker; lane 1, crude membrane (CM) from host AD1−8u⁻ cells, 8 μg; lane 2, CM from VyCDR1H cells expressing CDR1−ORF tagged with six histidines, 8 μg; lane 3, solubilized crude membrane fraction from VyCDR1H, 8 μg; lane 4, eluent from Ni^{2+}-NTA column with 500 mM imidazole, 1 μg; lane 5, 400 mM NaCl eluent from Q-Sepharose resin containing the pure Cdr1p, 1 μg. (B) Oligomycin-sensitive ATPase activity of purified Cdr1p. ATPase assay was performed by using purified Cdr1p as well as with different samples taken out during purification at 30 °C for 30 min as described in the methods section (numbers 1−5 show the same fractions as described in Figure 1A. The values are given as the means ± standard deviations (error bars) for three independent experiments). (C) Immunodetection of the purified Cdr1p. Panel a shows the detection with mouse monoclonal anti-penta His antibody (diluted 1:5000), and panel b shows the immunodetection of purified Cdr1p with rabbit polyclonal anti-Cdr1p antibody (diluted 1:1000). Numbers 1−5 show the loading pattern of different samples as described in Figure 1A.

Table 3: Minimum Inhibitory Concentration (MIC_{80}, μg/mL) of Cells Expressing Wild-Type Cdr1p and Its Mutant Variants

strains	Aniso	Cyclo	Flu	Mic
AD1−8u⁻	0.12	0.15	1	0.015
AD1002	16	1	64	1
VyCDR1H	16	1	64	2
SSCH−C193A	4	0.125	4	0.25
SSCH−W326A	16	0.5	32	1

Purified Cdr1p Elicits ATPase Activity. Purified Cdr1p was diluted with an equal volume of buffer E (without NaCl) and incubated with 0.01% (w/v) of lysolecithin (for 1 h at 4 °C), and then protein solution was incubated with SM2 Biobeads (Bio-Rad) to remove the detergent in the presence of lipids extracted from the host strain AD1−8u⁻. Finally, purified protein in the host lipid solution was sonicated in a water bath sonicator at 4 °C for 30 s. The aliquots of purified protein were stored at −80 °C until further use. It should be mentioned here that for lipid supplementation of purified Cdr1p, several lipid combinations of egg PC, egg PE, PS,

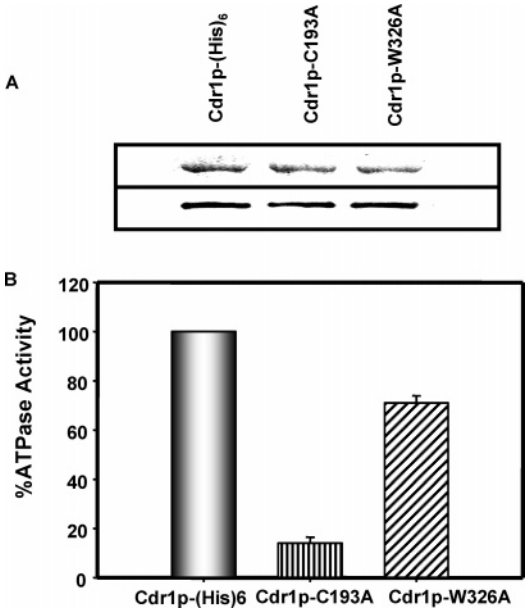

FIGURE 2: Mutant variants of Cdr1p and their ATPase activities. (A) Coomassie stained 8% SDS−PAGE purified wild-type and mutant variants (upper panel) [lane 1: purified wild-type Cdr1p; lane 2: purified mutant Cdr1p-C193A; lane 3: purified mutant Cdr1p-W326A] and immunodetection with mouse monoclonal anti-His antibody (lower panel). (B) Comparison of ATPase activity of purified Cdr1p with its purified mutant variants. ATPase activity of purified Cdr1p, Walker A mutant Cdr1p-C193A, and Walker B mutant Cdr1p-W326A were assayed, and each reaction was performed in triplicate; the values plotted (with ±SD) represent average of three independent experiments.

and cholesterol were tried, but the purified protein did not display specificity to any particular formulation of lipids. The restored ATPase activity with different lipid formulation was in the range of 1.13 −1.24 μmol (mg of protein)$^{-1}$ min^{-1} (data not shown). With increasing concentrations of lipid extracted from host strain AD1−8u$^-$, purified Cdr1p exhibited maximum ∼1.5-fold stimulation in ATPase activity (data not shown). For further analysis, the lipids extracted from the host strain in the ratio of 1:1 (lipid/protein) were routinely used.

The ATPase activity of purified Cdr1p was monitored by estimation of released inorganic phosphate (Pi). Figure 1B represents percentage of oligomycin-sensitive ATPase activity. It is evident that oligomycin-sensitive ATPase, which represents Cdr1p activity, increased in each step of purification and that purified Cdr1p shows maximum sensitivity to it (∼90%). As a control, when protein was heated at 95 °C for 10 min, the addition of ATP did not show any release of Pi and hence showed no activity (data not shown). These results were also confirmed by using a continuous cyclic method of ATPase assay (*22*) (data not shown). To ensure that the observed ATPase activity was not due to any contaminant, we used a mutated version Cdr1p-C193A, which has severely impaired ATPase activity owing to substitution of critical Cys193 of N-terminal nucleotide binding domain to Ala and mutant variant Cdr1p-W326A, which binds ATP poorly and elicits partially impaired ATPase activity (*14*−*16*). The purified Cdr1p-C193A protein (Figure 2A) predictably showed severely impaired activity, while purified mutant variant Cdr1p-W326A (Figure 2A) exhibited ∼70% ATPase activity as compared to wild-type protein. Thus, ATPase activities of purified mutant variants reconfirmed that the observed ATPase activity of purified Cdr1p is predominantly due to pure protein (Figure 2B).

Protein titration of purified Cdr1p mediated ATPase activity revealed that the increase in the rate of ATP hydrolysis was linear over a period of time and protein concentrations. Cdr1p mediated ATPase activity peaked over a broad pH range extending from 6.0 to 7.5 (data not shown) and was strongly dependent on the presence of divalent cations. The order of divalent cation dependence was observed to be $Mg^{2+} > Mn^{2+} > Ca^{2+} > Co^{2+}$ (Figure 3A).

Vanadate inhibits the ABC transporters by stabilizing a high-energy intermediate during the ATP hydrolysis cycle. It does so by replacing the terminal phosphate hydrolyzed by the ATPases in the occluded nucleotide state complex (*23*). We checked for its influence on Cdr1p and found (Figure 3B) that 0.5 mM vanadate could inhibit ∼50% of ATPase activity. The activity of purified Cdr1p was also inhibited by 10 mM of sodium azide (∼90%) and 50 μM of oligomycin (85−90%) (Figure 3B). NEM being a specific modifier for −SH groups affects ATPase activity of ABC transporters containing Cys residues, either in the nucleotide binding motifs or in the close proximity of the catalytic site of the proteins (*23*). In this study, we observed that NEM (0.5 mM) could reduce ATPase activity of purified Cdr1p by ∼70% (Figure 3B).

Cdr1p Is a General Ribonucleoside Triphosphatase. We also analyzed the substrate specificity of Cdr1p, and for this we checked the versatility of this class of protein in hydrolyzing different ribonucleotides independently. We observed that Cdr1p is capable of hydrolyzing all the four tested ribonucleotides if added separately i.e., ATP, UTP, GTP, or CTP (Figure 3C). Analysis of ATPase activity of Cdr1p revealed simple Michaelis−Menten kinetics with the K_M value ranging from 1.8 to 2.1 mM and V_{max} between 1.0 and 1.4 μmol (mg of protein)$^{-1}$ min^{-1} (LB plot is not shown). Kinetic analysis carried out with other NTPs indicated that Cdr1p has similar K_M values for ATP hydrolysis in the order of ATP > CTP > GTP > UTP (data not shown).

Intrinsic Trp Fluorescence of Purified Cdr1p Could Monitor ATP Binding. Exploiting the intrinsic fluorescence of Trp and Tyr residues of proteins has, for a long time, been relied upon as an efficient technique for studying protein−ligand complexes (*24*, *25*). Several groups have studied nucleotide binding by monitoring changes in the intrinsic fluorescence of the protein (*26*−*31*). We had earlier exploited intrinsic Trp fluorescence of the purified N-terminal domain of Cdr1p and demonstrated the role of conserved but uncommon Trp326 in nucleotide binding (*16*). Full protein Cdr1p contains 24 Trp residues, wherein several of them are located in extramembranous regions of the protein and thus are potentially highly accessible to their aqueous environment (Figure 4A). We explored whether intrinsic fluorescence of Trp(s) could be used to monitor nucleotide binding to purified Cdr1p. Purified and catalytically active Cdr1p upon excitation at 295 nm exhibited one sharp peak with an emission maximum at 330 nm, contributed by the Trp residues buried in a nonpolar environment, and another broad range peak between 340 and 360 nm, which is characteristic of solvent-exposed Trp(s) (Figure 4B, spectra 1). This clearly indicates that purified Cdr1p has two sets of Trp(s), wherein one set resides in a highly nonpolar environment and another set of Trp(s) is probably exposed to a polar

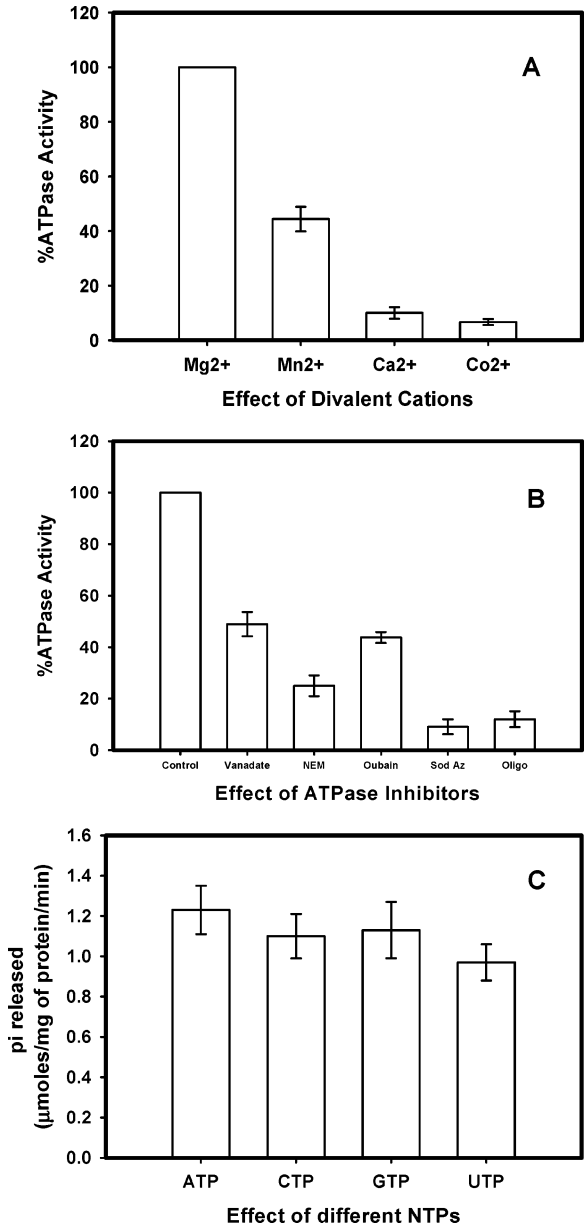

FIGURE 3: Biochemical characterization of the Cdr1p mediated ATPase activity. All of the experiments in this section have been performed in triplicate, and the values plotted (±SD) represent the average of three independent experiments. The assay conditions used for these experiments are similar to those described in the methods section except for the variations in biochemical parameters as follows. (A) Divalent cation dependent ATPase activity of purified Cdr1p. (B) Effects of ATPase inhibitors. The ATPase activity of purified Cdr1p was measured in the presence of various inhibitors, for example, sodium orthovanadate (0.5 mM) [it was prepared by boiling 50 mM solution in water for 3 min and the concentration was determined by molar absorbance (*14*)], *N*-ethylmaleimide (0.5 mM) [chemical modification of the protein by NEM was done by incubating the protein for 10 min at 25 °C in 100 μL of reaction mixture containing ATPase buffer (*14*)], sodium azide (10 mM), and oligomycin (50 μM) were added before initiation of the reaction. The bars represent the relative percentage decrease in the total ATPase calculated by taking the ATPase activity in the absence of inhibitors as 100%. (C) The ATPase assay was performed in the presence of 5 mM each NTP used in independent sets of assay mixture.

environment. Small aliquots of ATP added to the protein solution resulted in an increase in fluorescence emission intensity between 340 and 360 nm with a ΔF_{max} of ~70% (Table 4) in a concentration-dependent manner. This enhancement is saturable with a K_d of 1.7 ± 0.3 mM, which was in the range of measured K_M values for ATP hydrolysis. Upon the addition of ATP, the peak at 330 nm gets merged with the broad-spectrum peak (Figure 4B; spectra 2). After removing all the bound nucleotide by extensive dialysis of purified protein, we observed that Cdr1p regained its native conformation (data not shown). The reversal of enhanced fluorescence intensity to background level upon nucleotide removal suggests that the purified Cdr1p is well structured and undergoes reversible conformational changes upon ATP binding. To rule out the possibility of any artifactual measurements of intrinsic fluorescence upon nucleotide binding, we also monitored ATP binding in a purified mutant variant Cdr1p-W326A that is known to bind ATP very poorly (*16*) and another catalytically inactive Cdr1p-C193A with normal nucleotide binding. As expected, the ATP binding defective mutant variant Cdr1p-W326A showed very poor enhancement of intrinsic fluorescence intensity upon nucleotide addition (Figure 4C), whereas Cdr1p-C193A which displays normal ATP binding, exhibited conformational changes similar to wild-type purified Cdr1p (Figure 4C). These results of negative controls Cdr1p-W326A and Cdr1p-C193A confirmed that the observed enhancement of intrinsic fluorescence of purified Cdr1p upon ATP addition is indeed a consequence of nucleotide binding. Taken together, it is apparent that Cdr1p undergoes conformational changes upon ATP binding, which results in Trp(s) exposure to more hydrophobic environment.

TNP-ATP Mimics ATP Binding to Purified Cdr1p. To reconfirm intrinsic fluorescence data, we used TNP-ATP, a fluorescent ligand whose fluorophore has been used earlier as an extrinsic reporter for the binding process (*28*−*30*, *32*−*34*). Since TNP-ATP is excited at 408 nm, the emission spectrum obtained is entirely independent of contribution from intrinsic Trp fluorescence of the protein. The addition of increasing concentration of TNP-ATP to the purified protein markedly increased the ligand fluorescence and gave an emission maximum at 545 nm (Figure 4D; spectra 1−4). This enhancement in fluorescence intensity has also been observed previously for other proteins and has been attributed to an increase in quantum yields due to the more hydrophobic environment that the fluorophore encounters in the binding pocket. Figure 4E depicts the binding curves with increasing concentrations of TNP-ATP added to the purified native type Cdr1p and to the negative control protein Cdr1p-W326A. TNP-ATP binding with the purified Cdr1p gave a dissociation constant (K_d) of 6.27 ± 0.55 μM. As expected, purified Cdr1p-W326A which is impaired in ATP binding showed about 2-fold higher dissociation constant (11.45 ± 0.6 μM). It was possible to specifically compete out the bound TNP-ATP albeit only up to 40% by titrating it with ATP (data not shown), suggesting that the nature of binding of the two nucleotides in the same pocket may differ. As expected, no competition could be monitored for Cdr1p-W326A, which showed poor binding to TNP-ATP (data not shown).

Of note, ATP binding to purified Cdr1p is much weaker (K_d = 1.7 ± 0.3 mM) than that of TNP-ATP (K_d = 6.27 ± 0.55 μM), as reflected in a ~300-fold higher dissociation constant for the former in comparison to the latter. The fact that TNP-ATP has a substantially higher affinity for Cdr1p as compared to ATP itself suggests a probable stabilization of the ligand in the binding pocket via the trinitrophenyl

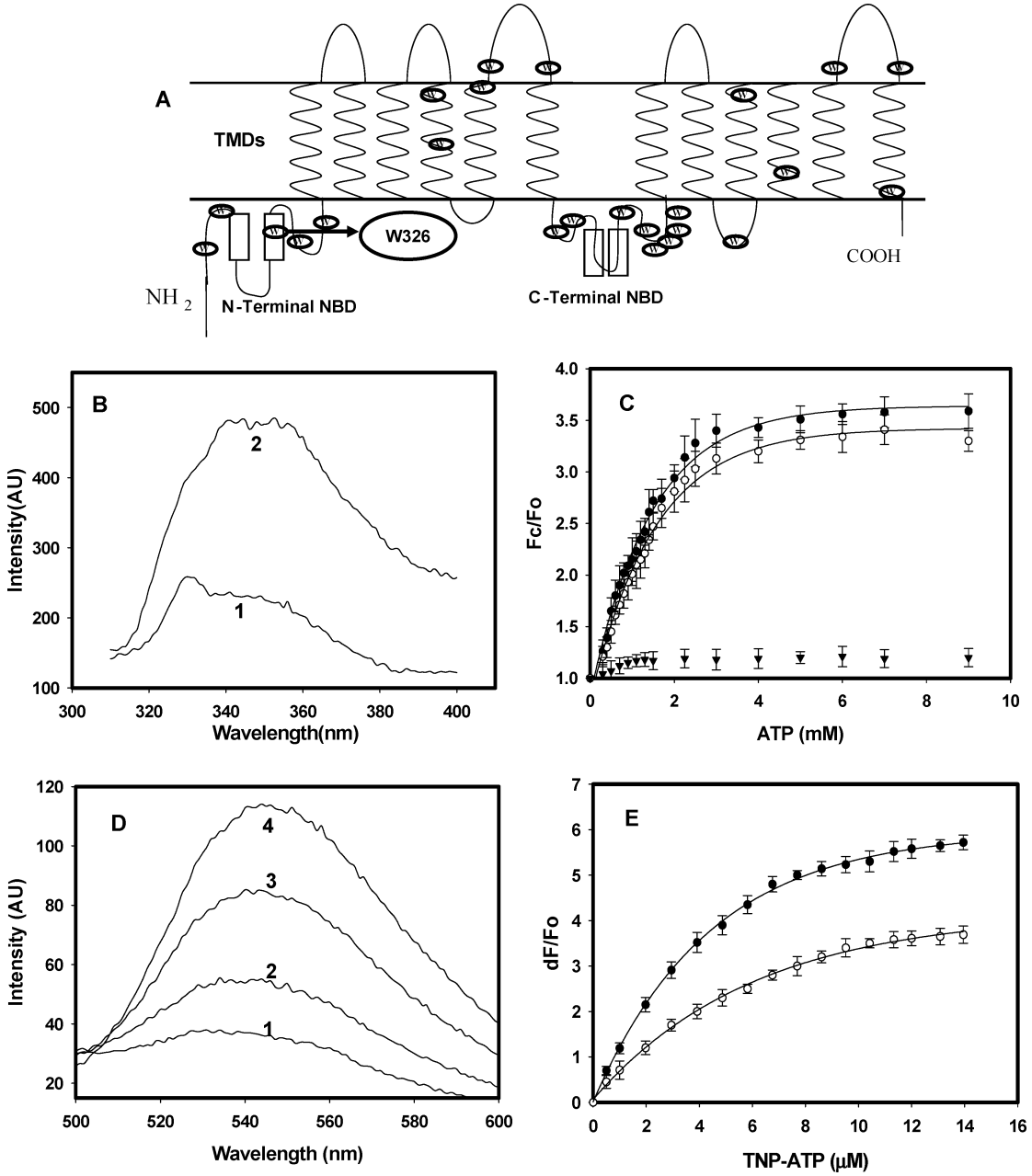

FIGURE 4: Intrinsic fluorescence measurements of Cdr1p. (A) Schematic representation of Cdr1p showing the locations of all the tryptophans. The predicted topology of full-length Cdr1p is shown here. All the 24 tryptophans are distributed throughout the protein. Five Trps in N-terminal NBD, 8 Trps in C-terminal NBD, 6 Trps in TMDs, 4 Trps in extracellular loops, and 1Trp in cytoplasmic loop. (B) ATP binding with purified Cdr1p monitored by enhancement in intrinsic fluorescence intensity. Samples containing 25 nM proteins in ATPase buffer were taken in a 1-cm path length cuvette and excited at 295 nm at room temperature (slit width 5 nm). Emission spectra were recorded between 310 and 400 nm (slit width 5 nm). Emission spectra were averages of five consecutive scans. Gradual addition of ATP with 1 min incubation for each addition gave the broad peak (between 340 and 360 nm), which we have monitored at 350 nm as the result of enhancement of intrinsic fluorescence intensity. Spectra 1 corresponds conformation without addition of ATP whereas spectra 2 shows final enhancement of intrinsic fluorescence intensity after addition of saturating concentrations of ATP. (C) Binding curves showing concentration-dependent binding of ATP to purified native Cdr1p (●), purified mutant proteins Cdr1p-C193A (○) and Cdr1p-W326A (▼). F_o refers to the fluorescence intensity of the sample in the absence of ATP, while F_c represents the fluorescence emission intensity at 350 nm upon ATP addition (corrected for dilution). The plot represents averages of three independent experiments done in triplicates. The bars represent standard deviations. (D) TNP-ATP binding with purified Cdr1p was monitored by extrinsic fluorescence**.** Binding with TNP-ATP was done as described previously. In brief, samples containing TNP-ATP in ATPase buffer were taken in a 1-cm path length cuvette and excited at 408 nm (slit bandwidth, 5 nm). Emission spectra were recorded between 500 and 600 nm (slit bandwidth, 5 nm). Spectra 1−4 show the enhancement of extrinsic fluorescence intensity upon addition of increasing concentrations of TNP-ATP with 0.5 μM (1), 2.5 μM (2), 7.5 μM (3), and 15 μM (4). (E) The plot represents binding of TNP-ATP to the purified native Cdr1p (●) and mutant protein Cdr1p-W326A (○). The values fitted in the curves are averages of three independent experiments done in duplicates. The bars represent standard deviations. The observed enhancement in K_d was statistically significant ($p < 0.01$).

moiety. The observation that ATP could not entirely compete out all of the bound TNP-ATP also indicated that the fluorescent analogue interacts with Cdr1p using its ATP moiety as well as the TNP group. Additionally, in contrast to ATP binding, the binding of its fluorescent analogue to Cdr1p is only marginally affected by the addition of EDTA,

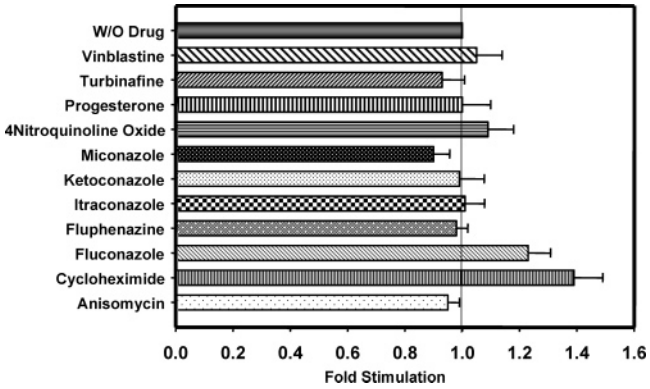

FIGURE 5: Effect of various drugs on the ATPase activity of purified Cdr1p. ATPase activity was performed in the presence of various drugs. Protein was preincubated with different drugs at 30 °C for 15 min before initiation of the reaction. Another set of reactions was done without preincubation with drugs and reactions were started for both the sets of experiments at same time with the addition of ATP. The bars represent the relative fold change in the total ATPase calculated by taking the ATPase activity in the absence of drugs as 1. Only fluconazole and cycloheximide exhibited very low levels (1.2- to 1.4-fold) of stimulation in ATPase activity. All the experiments were performed in triplicate, and the values plotted (±SD) represent the average of three independent experiments.

Table 4: Maximum Percentage Enhancement of Intrinsic Fluorescence Intensity {ΔF_{max}(%)} upon Ligand Binding

ligands	ΔF_{max}(%)
ATP	70 ± 4.5
cycloheximide	20 ± 3.5
fluconazole	28 ± 4.2
miconazole	15 ± 2.8
anisomycin	18 ± 2
ampicillin	2 ± 1

further suggesting that interaction of the TNP-ATP occurs mainly via its TNP moiety (data not shown). Such interactions and low K_d (high affinity) have been reported previously for TNP-ATP binding for several ABC as well as other proteins (*16*, *34*−*36*). Lansky et al. have shown that although ATP and TNP-ATP dock into the same nucleotide-binding pocket of Na^+/K^+ ATPase, they do so in different orientations and affinities (*34*).

Cdr1p Exhibits Moderate Activation of ATPase Activity by Drug Substrates. The mammalian ABC transporters such as mouse MDR3 and human P-gp have been very well demonstrated to elicit high drug stimulated ATPase activity (*32*, *37*, *38*). However, such dramatic stimulation of ATPase activity has so far not been demonstrated for any fungal ABC transporter. We monitored the ATPase activity in the presence of varying concentrations of different drugs and found that out of several drug substrates only fluconazole (150 μM) and cycloheximide (100 μM) could moderately stimulate the ATPase activity, which was up to 1.2- and 1.4-fold, respectively (Figure 5).

Intrinsic Fluorescence Measurements Reveal Specific Drug-Induced Changes in Cdr1p. As mentioned above, well-established drug stimulated ATPase activity of mammalian ABC transporters is not very well demonstrated in similar yeast proteins including in Cdr1p. Nonetheless, taking clues from other ABC proteins, cross talk between drug and ATP binding is expected to occur before Cdr1p could hydrolyze nucleotide to power the export of bound drugs. In the following experiments, we explored whether intrinsic fluorescence of Trp could also be exploited to dissect any interaction between NBDs and TMDs of Cdr1p upon drug binding. We observed that upon gradual addition of drugs such as cycloheximide, which is a well-known substrate of Cdr1p, there was a concentration-dependent enhancement of the intrinsic fluorescence intensity at 350 nm with ΔF_{max} of ~20% (Figure 6A). We also observed similar enhancement in the fluorescence intensity with other drug substrates as well, albeit to different levels (Table 4). Of note, the enhancement in intrinsic fluorescence upon the addition of various substrate drugs was always much less as compared to what we observed upon ATP addition. Interestingly, unlike Cdr1p substrate drugs, the addition of nonsubstrate drugs such as ampicillin did not bring about any change in intrinsic fluorescence intensity as it is evident from the ΔF_{max} of ~2% (Table 4). Of note, other nonsubstrates of Cdr1p such as methotrexate, mycophenolic acid, and crystal violet could not be tested for their nonresponsiveness since these drugs or their solvent used interfered with intrinsic fluorescence measurements (data not shown). ATP when added following addition of drug substrate resulted in further enhancement of fluorescence Trp intensity, which never exceeded ΔF_{max} of ~70% (Figure 6A). However, none of the substrate drugs could induce any further change in intrinsic fluorescence if added after the addition of saturating concentration of ATP (Figure 6B). It should be mentioned here that the addition of drug did not alter the affinity of Cdr1p for ATP, as it was evident from the K_d values for ATP binding in the presence of drug (~1.5 mM) and in the absence of drug (~1.7 mM).

Drug Binding to Cdr1p Is Independent of ATP Binding. The drug-induced changes in intrinsic fluorescence in catalytically inactive mutant variant, Cdr1p-C193A as well as in Cdr1p-W326A, remained unaffected (Figure 6C). Figure 6A depicts the change in intrinsic fluorescence upon the addition of drug followed by the addition of saturating concentration of ATP. It is apparent that like the native Cdr1p catalytically inactive Cdr1p-C193A, which shows normal ATP binding, addition of the nucleotide further enhanced intrinsic fluorescence. On the other hand, Cdr1p-W326A, which displays impaired ATP binding predictably, did not show any further enhancement in intrinsic fluorescence (Figure 6A).

DISCUSSION

In an effort to develop an understanding of the molecular details of drug binding and transport by the multidrug transporter Cdr1p, in this study we have attempted to isolate biochemical quantities of pure and active protein. Thus, we for the first time have generated the conditions for purification of homogeneous and active Cdr1p. The long-term exposure to the nonionic detergent *n*-dodecyl β-D-maltoside used for solubilization of overexpressed protein and its subsequent purification did not lead to any deleterious effect on Cdr1p activity. Our protocol resulted in more than 95% pure protein. The catalytic properties of the purified Cdr1p preparation were similar to those observed earlier by using plasma membrane fractions, indicating that the native conformation and function of the protein were preserved during purification (*10*). Our results clearly demonstrate that the purified Cdr1p behaves as general ribonucleotidase, which can hydrolyze ATP as well as other nucleotides such

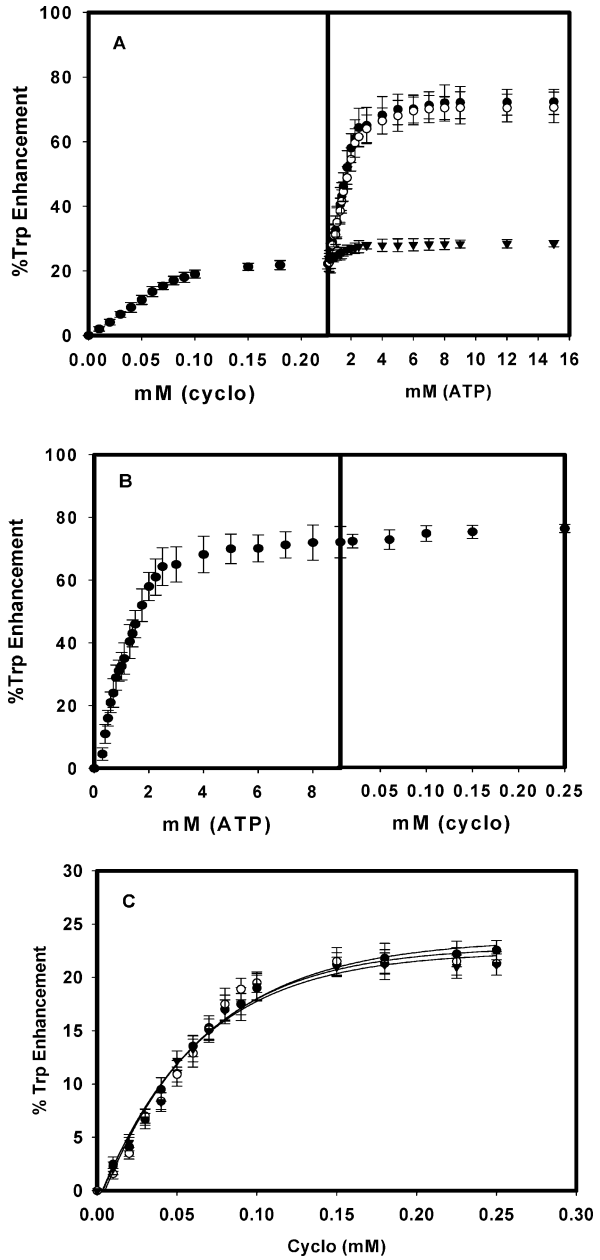

FIGURE 6: Intrinsic Trp fluorescence upon drug and ATP binding with purified wild-type Cdr1p and its mutant variants. (A) Binding curves show the % Trp enhancement in the intrinsic fluorescence intensity to the purified wild-type Cdr1p (●), mutant variants Cdr1p-C193A (○), and Cdr1p-W326A (▼) after addition of saturating concentrations of ATP, which was pretitrated with saturating concentrations of cycloheximide. (B) Binding curve shows the % Trp enhancement in the intrinsic fluorescence intensity of wild-type purified Cdr1p (●) after addition of saturating concentrations of drug, which was pretitrated with the saturating concentrations of ATP. The plot represents averages of three independent experiments done in triplicate. Bars show the standard deviation. (C) Binding curves showing % Trp enhancement upon concentration-dependent binding of cycloheximide to the wild-type purified Cdr1p (●) and mutant variants Cdr1p-C193A (○) and Cdr1p-W326A (▼).

as CTP, GTP, and UTP with almost equal affinity. This property of being an ATPase with wider nucleotide specificity is not uncommon for similar homologous proteins such as human CFTR and P-gp/MDR1 and for other yeast ABC proteins such as Pdr5p of *S. cerevisiae* (*39*) and CgCdr1p of *Candida glabrata* (*40*). The purified Cdr1p shows a K_M value in the range of 1.8 to 2.1 mM, which falls within the range of those values reported for other mammalian and fungal ABC transporters.

Unlike other mammalian ABC drug transporters, yeast proteins of this superfamily do not show dramatic drug stimulation of ATPase activity (*41*). Indeed, very few substrate drugs (present study) could stimulate Cdr1p activity up to only a maximum of ~1.4-fold. This probably relates to the placements of unique uncommon but conserved residues in N-terminal nucleotide binding domain of Cdr1p (*14*, *16*). The unique placements of amino acids such as Cys193 in Walker A and Trp326 in Walker B of N-terminal nucleotide binding domain and their involvement in ATP hydrolysis and binding, respectively, suggest basic mechanistic differences between fungal and mammalian ABC drug transporters. On the basis of the evidence from mammalian ABC exporters, it is assumed that the binding of the drug substrate to TMDs signals the stimulation of ATP hydrolysis at NBDs, which in turn initiate conformational changes resulting in translocation and release of the drug. However, in the absence of any significant drug stimulated ATPase activity, how Cdr1p and other fungal ABC transporters cross talk between TMDs and NBDs remains an interesting open question.

ATPase activity of human P-gp is strongly influenced by lipid environment (*42*). In contrast, our experiments show that Cdr1p does not prefer any particular lipid or its formulation. Cdr1p acts as a general phospholipid translocator (*43*), which transfers lipids from the inner monolayer to the outer monolayer (floppase). The translocase activity of Cdr1p may reflect its normal physiological role wherein it can conveniently export (flop) a large spectrum of drugs including lipids.

Our present study shows that upon ATP and drug binding, Cdr1p exhibits enhanced intrinsic tryptophan fluorescence intensity. In general, enhancement arises from a change in the local environment of the Trp probe, which can be either a result of conformational changes or directly by binding of the ligand close to the Trp residue. However, on the basis of our results, we cannot predict the closeness and accessibility of all 24 Trp(s) to the drug and nucleotide binding site. It should be pointed out that for human P-gp and other similar proteins where ligand binding results in quenching of Trp fluorescence (*44*) purified Cdr1p shows enhancement in intrinsic fluorescence.

By site-directed mutational analysis and intrinsic fluorescence measurements of a purified N-terminal NBD domain of Cdr1p, we had earlier shown that Cys193 is critical for ATP hydrolysis, while Trp326 is essential for ligand binding. The purified catalytically inactive Cdr1p-C193A exhibited ATP binding with K_d of ~1.9 mM, which was similar to native Cdr1p (K_d = ~1.7 mM). Taking mutant variant Cdr1p-W326A as a negative control for ATP binding, intrinsic fluorescence measurements predictably showed no significant binding of ATP. This reconfirmed that the changes in conformation monitored by intrinsic fluorescence with purified Cdr1p are not an artifact but are rather specific to nucleotide binding. Interestingly, drug binding to purified Cdr1p also induces change in conformation, which appears to be independent of nucleotide binding. These changes were very specific as nonsubstrate druglike water-soluble ampicillin was unable to produce any change in intrinsic fluorescence.

Table 5: Dissociation Constants of Drugs Binding to Purified Cdr1p and Its Mutant Variants

wild-type and mutant variants	cycloheximide (μM)	fluconazole (μM)
Cdr1p	28.3 ± 4.5	55 ± 6.3
Cdr1p−C193A	24.5 ± 3	58.5 ± 4
Cdr1p−W326A	30.5 ± 2.5	61 ± 5.5

Our results demonstrated that sequential addition of drugs and nucleotide resulted in ΔF_{max} values very similar to ATP alone, whereas after the addition of saturating concentrations of ATP none of the tested drug substrate could further enhance the intrinsic fluorescence intensity. Binding studies with purified catalytically inactive protein Cdr1p-C193A and Cdr1p-W326A demonstrated that drugs could bind to mutated proteins with the same affinities as that of wild-type (Table 5). These results indicate that the drug-induced conformational changes of Cdr1p are independent of ATP binding and hydrolysis. Of note, the enhancement in intrinsic fluorescence upon drug and ATP binding is not additive, implying that each binding induces specific and defined conformational changes.

In conclusion, we show that purified Cdr1p is catalytically active, which shows specific conformational changes upon ATP and drug binding. The nature of conformational changes upon drug and nucleotide binding remains to be resolved. By using purified N-terminal nucleotide binding domain, we had earlier reported that Trp326 of Walker B, out of all the five Trps (W101A, W148A, W326A W400A and W442A), is predominantly responsible for changes in intrinsic fluorescence upon nucleotide binding (*16*). However, from the results of the present study, we cannot predict the exact proximity of 24 Trps with regard to drug and nucleotide binding sites. The fact that the purified mutant variant Cdr1p-W326A (defective in ATP binding) which has all the rest of the 23 Trps intact, could not induce conformational changes upon nucleotide addition suggests that the Trp326 residue may be more important than the rest. However, Trp scanning of Cdr1p can only resolve such issues.

ACKNOWLEDGMENT

We thank R. D. Cannon for the plasmid and strains gifts. We are also thankful to Sneha Sudha Komath and Suresh V. Ambudkar for their helpful suggestions in preparation of the manuscript. We thank Ranbaxy laboratories limited, New Delhi, India, for providing fluconazole.

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BI0519147