Functional Characterization of N-Terminal Nucleotide Binding Domain (NBD-1) of a Major ABC Drug Transporter Cdr1p of *Candida albicans*: Uncommon but Conserved Trp326 of Walker B Is Important for ATP Binding†

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ABSTRACT: Using purified N-terminal NBD (NBD-512) domain of Cdr1p, a major multidrug extrusion pump of human pathogenic yeast *Candida albicans*, we show the relevance of the unique positioning of an atypical Trp326 residue. Similar to Cys193 in Walker A, Trp326 in the Walker B motif of Cdr1p is also a conserved feature of other fungal *A*TP *B*inding *C*assette (ABC) transporters. By employing fluorescence spectroscopy, chemical modification, and site-directed mutagenesis, we demonstrate that of the five Trp residues in the NBD-512 domain, Trp326 alone is important for nucleotide binding and subsequent conformational changes within the domain. Furthermore, mutation of Trp326 to Ala results in an increased K_M without appreciably affecting V_{max} of ATPase activity. Thus, Trp326 in NBD-512 appears to be important for nucleotide binding and not for its hydrolysis. Additionally, the role of Trp326 in ATP binding is independent of the presence of the adjacent well-conserved Asp327 residue which, like Cys193, has a catalytic role in ATP hydrolysis. Considering that Trp326 of Cdr1p is a typical feature of fungal transporters alone, our study suggests that these ABC transporters may reflect mechanistic differences with regard to nucleotide binding and hydrolysis as compared to their counterparts of non-fungal origin.

One of the most clinically significant mechanisms of azole resistance in the pathogenic yeast, *Candida albicans*, is overexpression of the multidrug transporter protein Cdr1p (Candida Drug Resistance), belonging to the ABC[1] (*A*TP *B*inding *C*assette) superfamily of transporters (*1*−*4*). 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 efflux this therapeutic azole and facilitate its survival (*5*−*11*). Thus, Cdr1p has not only acquired significant clinical importance but is also considered an important target in any design of strategies to combat antifungal resistance (*4*, *12*).

Typically, the predicted topology of Cdr1p exhibits features characteristic of an ABC transporter, namely, two highly hydrophobic transmembrane domains (TMDs) and two cytoplasmically localized nucleotide binding domains (NBDs). Each TMD comprises six transmembrane segments (TMS), which are envisaged to confer substrate specificity to Cdr1p. The substrates of Cdr1p vary enormously and include such structurally unrelated compounds as azoles, lipids, and steroids (*4*, *12*). 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 (*2*). Another important feature of these drug transporters is that they utilize energy from nucleotide hydrolysis to transport substrates across the plasma membrane against a concentration gradient. The conserved NBDs located at the cytoplasmic periphery are the hubs of such activity.

Like other ABC transporters, the NBDs of Cdr1p also have conserved Walker A, Walker B, and Signature C motifs that have been shown to be important for ATP hydrolysis (12−14). However, unlike ABC transporters of bacteria and mammals, the NBDs of *C. albicans* and other fungal ABC transporters have unique positioning of typical amino acids in the Walker A and Walker B motifs. The uncommon Cys193 of Walker A and Trp326 of Walker B in the N-terminal NBD of Cdr1p are two such examples. To examine the functional significance of these uncommon substitutions, we have recently demonstrated that replacement of the atypical Cys193 with Ala resulted in severely impaired ATPase activity of purified N-terminal NBD-512 (encompassing the first 512 amino acids of the protein) without any significant effect on ATP binding (*14*, *15*).

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[1] Abbreviations: ABC, ATP binding cassette; ATP, adenosine 5′-triphosphate; ATPase, adenosine-5′-triphosphatase; CFTR, cystic fibrosis transmembrane conductance regulator; EDTA, ethylenediaminetetraacetic acid; GST, glutathione *S*-transferase; IPTG, isopropyl thio-*β*-D-galactoside; kDa, kilodalton(s); NBS, *N*-bromosuccinimide; NBD, nucleotide binding domain; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TNP-ATP, 2′(3′)-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate; TMD, transmembrane domain; TMS, transmembrane segment; GFP, green fluorescent protein; ORF, open reading frame; PBS, phosphate buffer saline; PM, plasma membrane.

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In the present study, we have examined the significance of another uncommon but typical substitution, Trp326 of NBD-512, which is uniquely positioned in Walker B preceding a well-conserved Asp327. We show that, of the five tryptophan residues, only the uniquely positioned Trp326 of Walker B is important for ATP binding without being directly implicated in the catalytic activity of the purified NBD-512 domain. Further, we show that this effect is independent of the adjacent conserved Asp327 in the Walker B of NBD-512.

EXPERIMENTAL PROCEDURES

Materials. Ultrapure deoxyribonucleotides (dATP, dGTP, dCTP, and dTTP) for PCR, glutathione-sepharose 4B matrix, and the sequencing kit (Sequenase version 2.0 enzyme) were obtained from Amersham Biosciences Ltd. Ribonucleotides (ATP, CTP, GTP, and UTP), IPTG, protease inhibitors, TNP-ATP, miconazole, cycloheximide, anisomycin, oligomycin, and other molecular grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). NBS for tryptophan modification was obtained from Sisco Research Laboratories (Mumbai, India). The *Pfu* DNA polymerase for PCR amplification was purchased from Stratagene Inc, (La Jolla, CA). Oligonucleotides used in this study were commercially procured from Integrated DNA Technologies Inc. Fluconazole was kindly provided by Pfizer (Sandwich, Kent, United Kingdom) and Ranbaxy Laboratories (New Delhi, India). Anti-GFP monoclonal antibody was purchased from BD Biosciences Clontech (Palo Alto, CA). 8-Azido-[α-^{32}P]ATP was purchased from ICN Biomedical Inc. (Aurora, OH).

Methods. In Vitro Site-Directed Mutagenesis. All site-directed mutagenesis were carried out using a site-directed mutagenesis kit (Stratagene, La Jolla, CA) as previously described (*14*). The mutations W101A, W148A, W326A, W400A, and W442A were introduced into plasmid pSJGN1-8 according to the manufacturer's instructions. The forward primers used for mutagenesis were as follows: W101A (5′-AAAATTTCAATGCCAAATTTGCCGTTAAAAATTTA-AGAAAATTG-3′), W148A (5′-CTACTGTTACTAAT-GCTCTTGCCAAATAGCCACAGAAGGT-3′), W326A (5′-GGTGCTAATATCCAATGTGCCGATAATGCCACTAGA-GGG-3′), W400A (5′-TATTTTGAAAAGATGGGTGC-CAAATGTCCTCAAAGACAAACT-3′), W442A (5′-TCAA-GAATTTGAAACATATGCCAAAAATTC TCCCGAATAT-GCT-3′), D327N (5′-CTAATATCCAATGTTGGAATAAT-GCCACTAGAGGG-3′), and D327A (5′-CTAATATCCAAT-GTTGGGCAAATGCCACTAGAGGG-3′). The presence and authencity of the desired mutations were confirmed by dideoxy sequencing.

Overexpression, Purification and ATPase Assay of NBD-512 and Its Mutant Variant Proteins. The overexpressed wild-type NBD-512 and its mutant variant proteins were purified to homogeneity by our previously described procedure (*14*). The purity of the wild-type and mutant proteins was analyzed on SDS−PAGE, detected by Coomassie brilliant blue staining, and estimated to be over 95% pure. ATPase activity of the purified domains was determined by measuring the release of inorganic phosphate using a colorimetric assay (*14*).

Fluorescence Spectroscopic Measurements for Nucleotide Binding. Experiments were performed at room temperature in a 1 cm-path length cuvette using a Cary Eclipse Varian spectrofluorimeter with slit bandwidths of 5 nm for excitation as well as emission. All spectra were corrected for buffer fluorescence and for inner filter effects wherever required. Unless otherwise specified, 1 mL protein samples (0.4 μM) in 60 mM Tris-HCl, pH 6.5, 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 relation $F_c = F_{obs}\,\text{antilog}\{(OD_{ex} + OD_{em})/2\}$, where F_c represents the final corrected fluorescence intensity, F_{obs} is the observed fluorescence intensity already corrected for buffer contribution and dilution, OD_{ex} is the absorbance of TNP-ATP at 408 nm, and OD_{em} is its absorbance at 545 nm.

Nucleotide binding was monitored either by following quenching of intrinsic Trp fluorescence at 330 nm upon addition of ATP or enhancement of extrinsic fluorescence of TNP-ATP at 545 nm upon binding to protein.

NBS Modification. Samples were incubated with small aliquots of NBS (freshly recrystallized; stock concentration 5 mM) for 2 min at room temperature, and the intrinsic tryptophan fluorescence emission spectra were recorded. All spectra were corrected for dilution as well as for contribution from NBS. The decrease in fluorescence intensity at 330 nm was used to assess the extent of Trp modification for the protein. The modification reaction was also carried out at pH 4.6 in acetate buffer in keeping with the original method of Spande and Witkop (*16*) and found to give results comparable to those obtained at pH 6.5. Hence, all modification reactions described here correspond to those carried out at pH 6.5 since this was the pH at which nucleotide binding and ATPase activity assays for this protein were also carried out.

Intrinsic Fluorescence Quenching by Small Molecular Weight Quenchers. All fluorescence measurements were made at room temperature. Protein samples (0.4 μM) in buffer 60 mM Tris-HCl (pH 6.5) containing 8 mM $MgCl_2$ were titrated with small aliquots of a 5 M quencher (acrylamide, KI, and CsCl) solution. After correcting for dilution, we used the observed emission intensities at 330 nm to obtain Stern−Volmer plots (SV) as well as modified Stern−Volmer plots (*17*). For SV plots, data were plotted using the equation $F_o/F_c = (1 + K_{SV})[Q]$, where F_o and F_c represent the respective fluorescence intensities (corrected for dilution) in the absence and presence of quencher, $[Q]$ is the resultant quencher concentration, and K_{SV} is the SV quenching constant for the quencher. Modified SV plots were also plotted according to the equation $F_o/\Delta F = (f_a^{-1} + (K_a f_a)^{-1})[Q]^{-1}$ where, f_a is the fraction of accessible fluorophores and K_a is the corresponding SV quenching constant for this accessible fraction (*17*).

Site-Specific Mutagenesis and Development of Full Protein Cdr1p Mutant Variants. Site-directed mutagenesis was performed using the quick-change mutagenesis system. The

			Walker A	Walker B
C. albicans	Cdr1p(N)	187	GRPGAG**C**S--------323	IQC**W**DNATRGLD
C. albicans	Cdr2p(N)	185	GRPGAG**C**S------- 321	IQC**W**DNATRGLD
C. albicans	Cdr3p(N)	178	GRPGAG**C**S--------314	IQC**W**DNSTRGLD
C. albicans	Cdr4p(N)	190	GRPGAG**C**S--------326	VQC**W**DNSTRGLD
S.cerevisiae	Pdr5p(N)	193	GRPGSG**C**T--------329	FQC**W**DNATRGLD
S.cerevisiae	Snq2p(N)	192	GRPGAG**C**S--------330	IYC**W**DNATRGLD
C. glabrata	Phd1p(N)	186	GRPGSG**C**T--------329	FQC**W**DNATRGLD
C. neoformans	CnAfr1p(N)	258	GRPGAG**C**T--------394	VCS**W**DNSTRGLD
B. cinerea	Bmr1p(N)	195	GRPGAG**C**T--------331	VCA**W**DNSTRGLD
A. nidulans	AtrBp(N)	136	GRPGSG**C**T--------172	VFC**W**DNSTRGLD
A. fumigatus	AtrFp(N)	229	GRPGAG**C**S--------359	IVC**W**DNSTRGLD
H.sapiens	Pgp(N)	427	GNSGCG**K**S--------552	ILL**L**DEATSALD
H.sapiens	CFTR(N)	458	GSTGAG**K**T--------569	LYL**L**DSPFGYLD
H.sapiens	TAP1(N)	538	GPNGSG**K**S--------664	VLI**L**DDATSALD
H.sapiens	TAP2(N)	503	GPNGSG**K**S--------628	VLI**L**DEATSALD
E.coli	HlyA(N)	502	GRSGSG**K**S--------627	LLI**F**DEATSALD
S.typhimurium	HisP(N)	39	GSSGSG**K**S--------175	VLL**F**DEPTSALD
A. radiobacter	LacK(N)	36	GPSGCG**K**S--------155	VFL**F**DEPLSNLD
S.typhimurium	MalK(N)	36	GPSGCG**K**S--------155	VFL**L**DEPLSNLD

FIGURE 1: Sequence alignment of Walker A and B motifs from various ABC transporters. Comparison of the sequence alignment of Walker A and B residues in NBD-512 with those from other *N*-terminal nucleotide binding domains of some known ABC transporters. The conserved Cys and Trp residue in Walker A and B, respectively, of fungal NBDs and the equivalent Lys and Leu/Phe in nonfungal NBDs are shown in bold. The conserved Asp residue adjacent to the Trp NBD1 from Cdr1p is underlined.

mutations were introduced into plasmid pPSCDR1-GFP according to the manufacturer's instructions, and DNA sequencing of the ORF confirmed the desired nucleotide sequence alteration. The mutated plasmid pPSCDR1-GFP, after linearizing with *Xba*I, was used to transform AD1-8u$^-$ cells, for uracil prototrophy by the lithium acetate transformation protocol (*18, 19*).

RESULTS

Intrinsic Tryptophan Fluorescence of NBD-512 Monitors ATP Binding. Exploiting the intrinsic fluorescence of Trp and Tyr residues of proteins has, for long, been relied upon as an efficient technique for studying a protein−ligand complex (*17*). Thus, several groups have studied nucleotide binding by monitoring changes in the intrinsic fluorescence of the protein (*20*−*26*). We too observed that ATP binding by purified NBD-512 could be monitored using the intrinsic tryptophan fluorescence of the protein. Interestingly, sequence analysis of NBD-512 revealed that it has five Trp residues of which Trp326 is uniquely positioned in its Walker B motif and could be in a position to monitor approach of the ligand. Further, this Trp residue is conserved in other fungal ABC transporters as well (Figure 1). We therefore undertook a detailed analysis of the explicit role of this and other Trp residues in the functionality of the N-terminal NBD of Cdr1p using a combination of site-directed mutagenesis and fluorescence spectroscopy.

Upon excitation at 295 nm, the NBD-512 domain exhibits a spectrum with an emission maximum at 330 nm, characteristic of proteins with significant content of buried Trp residues (Figure 2A, panel a) (*17*). Small aliquots of ATP added to the protein solution result in quenching of the fluorescence emission from NBD-512 in a concentration dependent manner (Figure 2B). This quenching is saturable with a K_d of 76.0 ± 4.0 μM (Figure 2B, inset).

ATP binding to NBD-512 is Mg^{2+}-dependent since it is possible to reverse the fluorescence quenching observed upon nucleotide binding by titrating with EDTA (Figure 2A, panel b). As can be seen from Figure 2A, panel b, addition of EDTA causes a small quenching in the fluorescence emission of NBD-512 (curve 2). But addition of 1 mM ATP does not cause any further quenching of the fluorescence emission (curve 3), suggesting that ATP does not bind to NBD-512 in the presence of EDTA. However, in the presence of a large excess of $MgCl_2$, ATP binds to NBD-512 and quenches its intrinsic fluorescence (curve 4). Thus, Mg^{2+} seems to be important for ATP binding by NBD-512.

In the present study, we also observed that nucleotides such as CTP, GTP, and UTP could cause similar extents of intrinsic fluorescence quenching with purified NBD-512 domain and had comparable affinities for the protein (data not shown). This is consistent with our earlier observation that isolated NBD-512 domain elicits general ribonucleotidase activity wherein nucleotides other than ATP also show significant binding and hydrolysis (*14*).

Mg-ATP Protects Trp Residue(s) of NBD-512 from NBS Modification. Mg-ATP-dependent quenching of intrinsic fluorescence (Figure 2A,B) suggests a possibility that one or more Trp residues are present at or in close proximity to the nucleotide-binding site of NBD-512.To test this hypothesis, side group specific modification was carried out by using the Trp specific reagent, *N*-bromosuccinimide (NBS). NBS specifically and irreversibly oxidizes the indole moiety of accessible Trp residues in the protein to the oxindole derivative which, being nonfluorescent, results in quenching of the fluorescence emission spectrum of the protein (*16*). NBS treatment of NBD-512 quenches nearly 60% of its Trp emission (Figure 2C). The lack of complete fluorescence quenching upon NBS addition suggests that all five Trp residues of NBD-512 are not equally accessible to the modifying reagent. The shape of the spectrum also remains unchanged (spectra not shown), suggesting that chemical modification does not result in denaturation or overall conformational change in the domain. Significantly, 5 mM

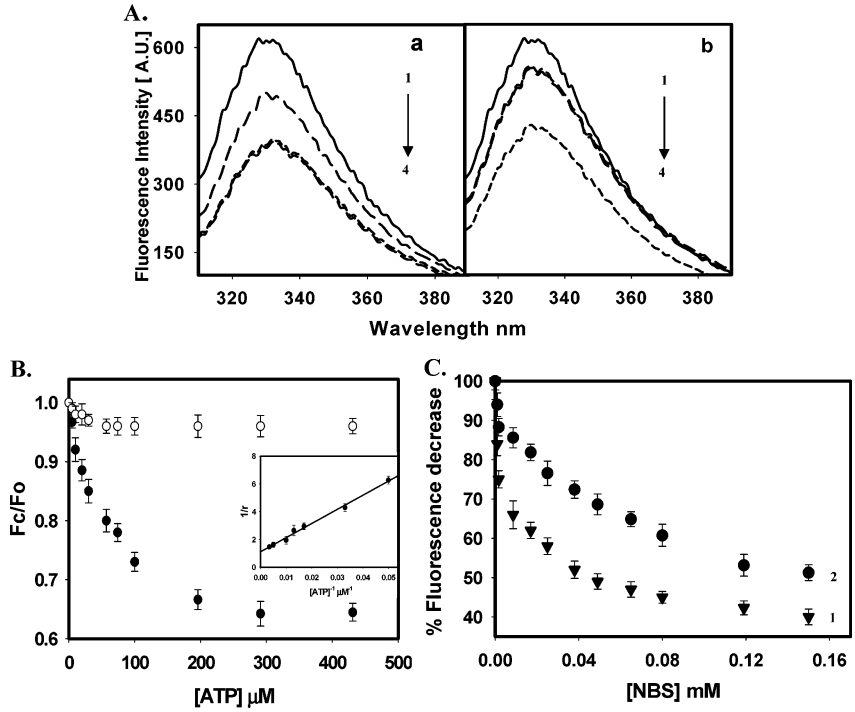


FIGURE 2: ATP binding with purified NBD-512 protein monitored by intrinsic Trp fluorescence. Samples containing 0.4 μM NBD-512 in ATPase buffer were taken in a 1 cm path length cuvette and excited at 295 nm at room temperature (slit bandwidth 5 nm). Emission spectra were recorded between 310 and 400 nm (slit bandwidth 5 nm). All emission spectra shown are averages of 10 consecutive scans and corrected for dilution. (A, panel a) Fluorescence emission spectra of NBD-512 measured both in the absence (curve 1) and in the presence of 0.08 mM (curve 2), 0.2 mM (curve 3), and 1.0 mM (curve 4, overlaid on curve 3) ATP. (A, panel b) Curve 1 represents the fluorescence emission spectrum of NBD-512 measured in the absence of EDTA. Curve 2 shows the small decrease in fluorescence intensity of the protein in the presence of 8 mM EDTA. There was no further decrease in fluorescence intensity upon addition of 1 mM ATP (curve 3, overlaid on curve 2). However, when 100 mM Mg^{2+} is added to this sample of protein, containing 8 mM EDTA and 1 mM ATP, the extent of fluorescence quenching is increased (curve 4). (B) Binding curve showing concentration dependent binding of ATP to purified NBD-512 in the absence (●) and the presence (○) of 8 mM EDTA. Normalized fluorescence intensities (F_c/F_o) were plotted as a function of ATP concentration, where F_o refers to the fluorescence intensity of the sample in the absence of ATP, while F_c represents the fluorescence emission intensity at 330 nm upon ATP addition (corrected for dilution). The plot represents averages of three experiments done in triplicates. The bars represent standard deviations. From the slope and ordinate of the Scatchard plot shown in the inset, the dissociation constant (K_d) for ATP binding was determined to be 76.0 ± 4.0 μM, assuming a single binding site per NBD-512 molecule. (C) Partial protection of Trp residues of NBD-512 from modification by NBS in the presence of 5 mM ATP. Increasing concentration of NBS causes specific and saturable modification of Trp residues of NBD-512 (curve 1). In the presence of ATP, however, the extent of this modification is much less (curve 2).

ATP partially protects the protein from modification by NBS as evidenced by a reduction in fluorescence quenching (Figure 2C), suggesting that ATP prevents access to one or more Trp residues of NBD-512 by the group-specific reagent.

Trp326 Monitors ATP Binding in NBD-512. The above results suggest that specific Trp residue(s) of NBD-512 could play a role in nucleotide binding by the protein. To confirm this as well as to examine which of the five Trp residues could be involved in nucleotide binding, we used site-directed mutagenesis to mutate each of the five Trp residues of NBD-512 and replaced them with Ala (Figure 3A). The mutated protein variants designated as NBD-512 W101A, NBD-512 W148A, NBD-512 W326A, NBD-512 W400A, and NBD-512 W442A were overexpressed and purified in a manner similar to NBD-512 (*14*). Each of the purified mutant proteins migrated as a single homogeneous band on SDS−PAGE with the anticipated M_r of ~57 kDa (Figure 3B, top panel). We monitored ATP binding of the individual mutant proteins using intrinsic fluorescence measurements in a manner similar to that used for NBD-512. As can be seen from Table 1, the mutant proteins NBD-512 W101A, NBD-512 W148A, NBD-512 W400A, and NBD-512 W442A displayed ATP-dependent quenching of intrinsic fluorescence and ATP binding comparable to those of NBD-512. On the other hand, NBD-512 W326A experienced negligible quenching of intrinsic fluorescence upon titration with ATP. Of note, therefore, poor quenching in NBD-512 W326A did not permit determination of K_d. Notably, addition of EDTA did not cause any change in the fluorescence emission of NBD-512 W326A in the presence of ATP (data not shown). Hence, the fluorescence quenching observed with EDTA for ATP binding to native NBD-512 protein (Figure 2A, panel b) must be specifically related to the presence of Trp326 in its Walker B motif. Thus, it is apparent that Trp326 alone appears to be monitoring nucleotide binding in the isolated domain. The ATPase activity of the individual mutant proteins is shown in Figure 3B, bottom panel (discussed later).

NBD-512 W326A Shows Reduced Binding to TNP-ATP. The fact that, of all the mutant proteins, NBD-512 W326A alone exhibits reduced intrinsic fluorescence quenching upon ATP binding, led us to ask whether the effect is truly due to a diminution in affinity of the mutant protein for the nucleotide or whether it is merely a consequence of the mutation removing our reporter group from the site. In other words, it is conceivable that ATP continues to bind normally to NBD-512 W326A but our technique fails to detect it since a nonfluorescent Ala residue has taken the place of Trp at this site. To verify the actual cause behind this drastic

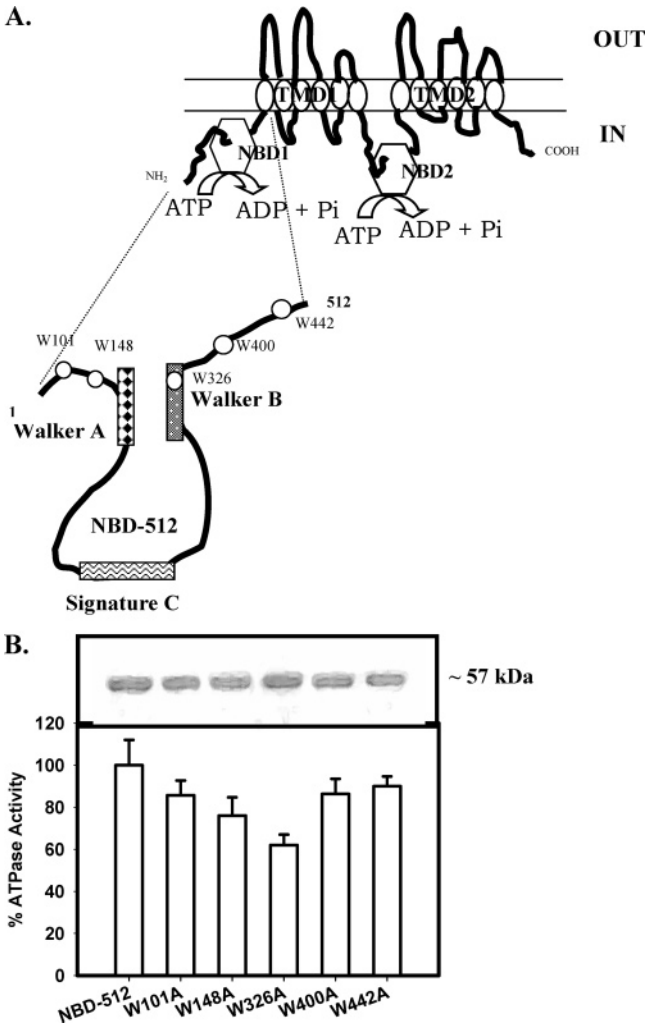


FIGURE 3: Trp replacement mutants of NBD-512. (A) Schematic representation of Cdr1p and NBD-512. The predicted topology of the nucleotide binding and transmembrane domains of the full Cdr1p is shown here. The 1−512 amino acid polypeptide corresponds to N-terminal nucleotide binding domain (NBD-512) of Cdr1p. The locations of the Trp residues mutated and investigated in this study, namely, W101, W148, W326, W400, and W442 are indicated. (B) SDS−PAGE and ATPase activity of NBD-512 and the five Trp mutants utilized in this study. Upper panel shows SDS−PAGE of wild-type and mutant proteins following purification (described in Experimental Procedures). Aliquots (5 μg) of each protein were analyzed on a 10% SDS−PAGE gel (*51*) and detected by Coomassie Blue R-250 (*51*, *52*). Lower panel shows a comparison of ATPase activity of purified NBD-512; each experiment had reactions performed in triplicates, and the values plotted represent the average of three such independent experiments. The bars represent standard deviations. The *p*-values for NBD-512 W101A (*p*-value 0.05), NBD-512 W148A (*p*-value 0.08), NBD-512 W326A (*p*-value 0.006), NBD-512 W400A (*p*-value 0.097), and NBD-512 W442A (*p*-value 0.06) were determined and were not statistically significant.

reduction in intrinsic fluorescence quenching upon ATP binding to NBD-512 W326A, we used a fluorescent ligand (TNP-ATP) whose fluorophore has been used earlier as an extrinsic reporter for the binding process (*22*, *23*, *27*−*31*). As has been previously reported by us, the addition of TNP-ATP to NBD-512-purified protein resulted in an enhancement of the ligand fluorescence (*14*). This enhancement in fluorescence intensity has also been previously observed for other proteins and has been attributed to an increase in quantum yields due to the more hydrophobic environment

Table 1: Dissociation Constants (K_d) Obtained from Intrinsic Fluorescence Quenching upon ATP Binding to NBD-512 and Its Mutant Variants

	quenching (%)	K_d (μM)
NBD-512	35 ± 6	76 ± 4
NBD-512 W101A	31 ± 4	86 ± 6
NBD-512 W148A	30 ± 3	170 ± 9
NBD-512 W326A	2 ± 3	*a*
NBD-512 W400A	33 ± 5	177 ± 6
NBD-512 W442A	32 ± 4	172 ± 8
NBD-512 D327A	30 ± 5	91 ± 4
NBD-512 D327N	31 ± 4	86 ± 2

[a] Dissociation constant could not been determined due to poor intrinsic fluorescence quenching. With the exception of NBD-512 W326A, the *p*-values for all mutants have been calculated and none were statistically significant: W101A (*p*-value 0.057), W148A (*p*-value 0.23), W400A (*p*-value 0.197), W442A (*p*-value 0.07), D327N (*p*-value 0.07), and D327A (*p*-value 0.127). The values (with ±SD) represent the average of three independent experiments done in triplicates.

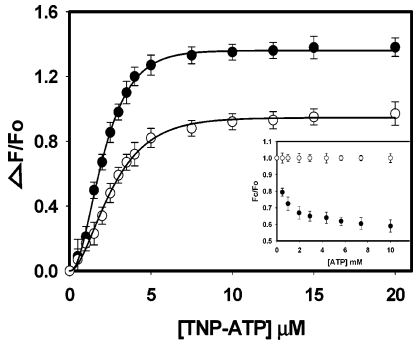


FIGURE 4: TNP-ATP binding with purified NBD-512 and mutant NBD-512 W326A protein monitored by extrinsic fluorescence. Samples containing TNP-ATP in ATPase buffer were taken in a 1 cm path length cuvette and excited at 408 nm at room temperature (slit bandwidth 5 nm). Emission spectra were recorded between 500 and 600 nm (slit bandwidth 5 nm). Binding curve showing concentration dependent binding of TNP-ATP to purified NBD-512 (●) and mutant NBD-512 W326A (○) protein. Increasing concentrations of TNP-ATP, as indicated in the figure, were incubated with 0.4 μM protein, and fluorescence emission spectra were recorded between 500 and 600 nm (room temperature). Normalized difference in fluorescence intensities ($\Delta F/F_o$) were plotted as a function of TNP-ATP concentration, where ΔF represents the change in fluorescence emission intensity (corrected for dilution and inner filter effects as described under Experimental Procedures) at 545 nm upon protein addition, while F_o refers to the intensity of the original sample in the absence of protein. The plot represents averages of three experiments done in triplicates. The bars represent standard deviations. The dissociation constants (K_d) were determined to be 1.85 ± 0.13 μM for NBD-512 and 3.16 ± 0.12 μM for NBD-512 W326A. Inset: displacement of TNP-ATP from NBD-512 and its mutant NBD-512 W326A by ATP. TNP-ATP (20 μM) was incubated with 0.4 μM protein NBD-512 (●) or NBD-512 W326A (○) in ATPase buffer. Once the fluorescence signal was steady, ATP was added in small increments, as shown, and the change in fluorescence intensity was measured 3 min after each addition. The results shown are averages of three different experiments done in triplicates. The bars represent standard deviations.

that the fluorophore encounters in the binding pocket (*22*, *23*, *27*−*30*). It is possible to specifically compete out the bound TNP-ATP by titrating with ATP (Figure 4 and inset), suggesting that the two nucleotides bind in the same pocket. The dissociation constant for the interaction was calculated from the data which fitted well with the equation suggested by Kubala et al. (*31*) for a model with a single binding site per protein molecule. However, binding of ATP to NBD-

512 is much weaker (K_d 76.0 ± 4.0 μM) than that of TNP-ATP (K_d 1.85 ± 0.13 μM), as reflected in a 41-fold higher dissociation constant for the former in comparison to the latter.

The fact that TNP-ATP has a substantially higher affinity for NBD-512 as compared to ATP itself suggests a probable stabilization of the ligand in the binding pocket via the trinitrophenyl moiety. That ATP could not entirely compete out all of the bound TNP-ATP also indicated that the fluorescent analogue interacts with NBD-512 using its ATP moiety as well as the TNP group (Figure 4). Additionally, in contrast to ATP binding, the binding of its fluorescent analogue to NBD-512 is only marginally affected by the addition of EDTA, further suggesting that the interaction of TNP-ATP occurs mainly via its TNP moiety (data not shown). Such interactions have been reported previously for TNP-ATP binding to Na^+/K^+ ATPase also. Using mutational analysis, Lansky et al. have shown that although ATP and TNP-ATP dock into the same nucleotide binding pocket of this enzyme, they do so in different orientations (*30*).

Significant enhancement in the fluorescence of TNP-ATP is also observed upon binding to any of the four mutant variants NBD-512 W101A, NBD-512 W148A, NBD-512 W400A, and NBD-512 W442A (data not shown), comparable to that obtained for the isolated native NBD-512 domain, indicating that these four mutants are not altered in their interaction with TNP-ATP either. However, the increase in fluorescence intensity upon TNP-ATP addition to NBD-512 W326A is appreciably much less than that for NBD-512 (spectra not shown). Notably, unlike in the case of NBD-512, TNP-ATP binding to NBD-512 W326A could not be competed out with ATP (Figure 4, inset). It appears therefore that TNP-ATP predominantly interacts with NBD-512 W326A via its trinitrophenyl moiety and not through ATP, reconfirming the hypothesis that Trp326 may be important for the docking of ATP in the binding pocket.

Additionally, we used a radiolabeled analogue, 8-azido-[α-^{32}P]ATP, to further confirm the poor ligand binding behavior of NBD-512 W326A. We observed that compared to wild-type NBD-512, mutant variant NBD-512 W326A required approximately 5-fold higher concentration of the ligand to show proportional extent of binding to the ligand (data not shown). Thus, by three different methods, we confirmed that the mutant variant NBD-512 W326A indeed showed reduced affinity for ATP and its analogues as compared to native NBD-512. Since a precise K_d value for ATP binding by this mutant could not be calculated from any of these experiments, we estimated the extent of this interaction from K_M values calculated for ATP hydrolysis by this protein (discussed below).

ATP Fails To Protect NBD-512 W326A from Modification by NBS. As mentioned earlier, the presence of 5 mM ATP partially protects NBD-512 from NBS modification (Figure 2C). We argued that if Trp326 alone is involved in ATP binding then ATP should not be able to protect any of the Trp residues of NBD-512 W326A from side group specific modifications. Expectedly, when NBS modification of NBD-512 W326A is carried out in the absence or presence of ATP, the same extent of fluorescence quenching is observed (~54%, figure not shown). This implies that ATP does not shield any other Trp residue of the protein and the partial protection of NBD-512 from NBS modification by ATP is indeed due to shielding of Trp326 of the protein.

Replacement of Trp326 by Alanine Results in an Increase in K_M for ATPase Activity. Of note, though NBD-512 W326A is considerably affected in its ability to bind ATP, a similar drastic effect is not immediately apparent in its ability to hydrolyze ATP, from Figure 3B, bottom panel. However, Lineweaver−Burk analyses showed that NBD-512 W326A has a 5-fold higher K_M (600 ± 15 μM) as compared to that of NBD-512 (120 ± 4 μM) (plots not shown). The V_{max} values of the two proteins vary by less than 2-fold (136.27 ± 4 nmol min^{-1} mg protein^{-1} in the case of NBD-512 W326A and 76.80 ± 6 nmol min^{-1} mg protein^{-1} in the case of NBD-512). Thus, although NBD-512 W326A is able to bind the substrate, it does so more weakly than NBD-512 itself. We also point out that in our previous paper (*14*) the range of K_M for NBD-512 was erroneously reported as 0.8−1.0 mM. It should have actually read 0.08−0.1 mM.

It must be also pointed out that, unlike in the binding experiments, the substrate concentration used for monitoring ATPase activity in Figure 3B, bottom panel, is far in excess of the K_M value so as to get a reliable estimate of the V_{max} value. At these high concentrations, NBD-512 W326A is able to bind ATP. Once docked in the right orientation, the substrate is hydrolyzed if the catalytic residues of the molecule remain unaltered.

ATP Binding Leads to Conformational Changes in NBD-512. Having thus identified that Trp326 is important for ligand binding, we examined if there are conformational changes associated with nucleotide binding by the molecule. This is particularly significant since the cross talk between the NBDs of the ABC transporter and its TMDs are presumed to be critical for drug-transport by these efflux proteins (*32*−*35*).

Steady-state intrinsic fluorescence quenching has been used extensively to study dynamic conformational changes in proteins and to assess subtle changes in the environment of Trp residues (*17*, *36*−*38*). We observed that acrylamide, unlike other quenchers, had complete accessibility to all Trp residues of NBD-512 in the absence or presence of ATP (Table 2). Hence, it could not be used to provide information about conformational changes in NBD-512 upon nucleotide binding. Therefore, we employed I^- and Cs^+, charged quenchers which would primarily probe solvent accessible Trp residues, to obtain information regarding conformational changes in NBD-512 upon ATP binding (*17*, *36*).

Fluorescence Quenching with Iodide. When purified NBD-512 domain alone was titrated with I^-, we obtained biphasic Stern−Volmer plots implying that solvent accessible Trp residues are more readily quenched by the charged quencher than are the buried ones (Figure 5A). Titration by I^- resulted in approximately 86% of the Trp residues being accessible to the quencher. It is particularly interesting that f_a is nearly 100% for I^- in the presence of Mg-ATP (Table 2). This is also evidenced by the near-linear Stern−Volmer plot obtained for this quencher in the presence of ATP. Thus, clearly, ATP binding results in significant conformational changes in NBD-512 that alter the accessibility of its Trp residues to I^-.

The mutant protein NBD-512 W326A, on the other hand, showed no difference in accessibility to I^- in the absence (f_a = 24.0%) or presence of Mg-ATP (f_a = 25.8%) (Table

Table 2: Summary of Parameters Obtained from Intrinsic Fluorescence Quenching for NBD-512 and Its Mutant Variants Using Small Molecular Weight Quenchers[a]

parameters	maximal quenching (%)	K_{SV1} (M^{-1})	K_{SV2} (M^{-1})	f_a (%)	K_a (M^{-1})
		NBD-512			
acrylamide	100 ± 3	9 ± 1.1	NR[b]	97.7 ± 4.1	9.4 ± 1.2
acrylamide + 5 mM ATP	100 ± 2	6.6 ± 0.9	NR[b]	107 ± 2.1	6.7 ± 0.9
acrylamide + 150 mM NaCl	100 ± 0.0	7.2 ± 0.8	NRb	107.0 ± 2.3	7.2 ± 1.1
KI	82.4 ± 2	6.7 ± 1.3	4.8 ± 1.9	86.0 ± 2.7	13.7 ± 0.5
KI + 5 mM ATP	100 ± 1	7.9 ± 0.1	NR[b]	98.0 ± 0.9	9.4 ± 1.7
KI + 150 mM NaCl	85.4 ± 5	8.9 ± 0.6	5.9 ± 0.7	87.8 ± 1.7	19.5 ± 1.4
CsCl	50.6 ± 2	4.2 ± 0.7	0.8 ± 0.1	32.1 ± 2.1	17.3 ± 2.9
CsCl + 5 mM ATP	54.5 ± 3	3.6 ± 0.3	0.9 ± 0.1	47.3 ± 1.9	9.2 ± 2.1
CsCl +150 mM NaCl	54.2 ± 5	2.4 ± 0.6	0.8 ± 0.3	43.1 ± 1.5	7.5 ± 1.7
		NBD-512 W326A			
KI	25.6 ± 1	3.6 ± 1.3	0.4 ± 0.1	24.0 ± 1.1	4.0 ± 2.9
KI + 5 mM ATP	24.9 ± 2	3.3 ± 1.5	0.3 ± 0.1	25.8 ± 2.4	4.2 ± 2.9
CsCl	13.6 ± 1	0.6 ± 0.1	0.2 ± 0.1	13.3 ± 2.1	5.0 ± 2.9
CsCl + 5 mM ATP	13.0 ± 1	0.6 ± 0.2	0.18 ± 0.2	15.0 ± 2.2	5.6 ± 2.9
		NBD-512 C193A			
KI	80 ± 5	5.3 ± 1.9	3.8 ± 2.3	85 ± 1.7	11.7 ± 2.5
KI + 5 mM ATP	100 ± 1	9.0 ± 1.6	NR[b]	96 ± 0.7	9.9 ± 1.4
CsCl	50 ± 3	4.2 ± 1.8	1.2 ± 0.9	34.1 ± 2.1	17.5 ± 2.9
CsCl + 5 mM ATP	55 ± 5	3.3 ± 2.9	1.8 ± 0.9	51.7 ± 1.2	7.7 ± 1.7
		NBD-512 D327N			
CsCl	51 ± 2	4.6 ± 0.25	1.9 ± 0.3	35.4 ± 2.1	6.8 ± 2.5
CsCl + 5 mM ATP	54 ± 4	3.8 ± 0.5	0.9 ± .5	46.8 ± 1.5	6.47 ± 2.8

[a] K_{SV1} and K_{SV2} are Stern−Volmer (SV) quenching constants for each linear portion of a biphasic SV plot, f_a is the fraction of accessible fluorophores, and K_a is the corresponding SV quenching constant for this accessible fraction obtained from modified Stern−Volmer plots (*17*, *38*). The maximal quenching reported for the proteins in the presence of ATP is that observed for the protein−ligand complex rather than that observed for the free protein. For further details see Experimental Procedures. The values (with ±SD) represent the average of three independent experiments done in triplicates. [b] NR, not required since the Stern−Volmer plots were linear.

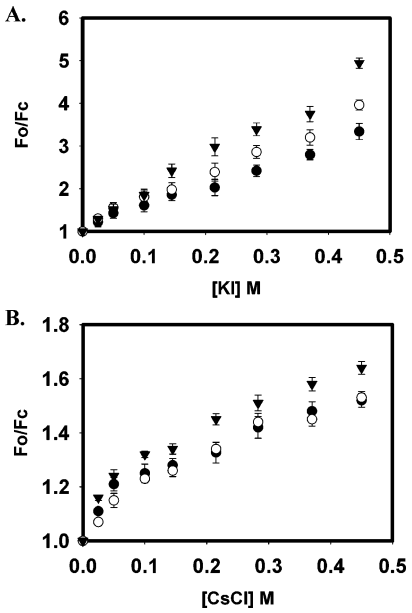


FIGURE 5: Intrinsic fluorescence quenching of wild-type NBD-512 using different quenchers. Quenching was carried out using 0.4 μM of isolated NBD-512 protein in the absence (●), presence of 150 mM NaCl (○), or presence of 5 mM ATP (▼) over a range of 0−0.45 M of quencher at room temperature (*17*, *36*−*38*). (A) Stern−Volmer plots for quenching with KI. (B) Stern−Volmer plots for quenching with CsCl. Representative Plots of data obtained in three different experiments done in duplicates are shown. The bars represent standard deviation.

2). It must be reemphasized that NBD-512 W326A does not have a Trp residue in the proposed ATP-binding pocket, and therefore, any change in intrinsic fluorescence would reflect changes in the environment of other Trp residues outside the nucleotide-binding pocket. Clearly, the presence of Mg-ATP does not alter the accessibility of I^- to the Trp residues of NBD-512 W326A. In other words, the other four Trp residues of this mutant protein do not experience any change in their environment in the presence or absence of the nucleotide. This suggests that the mutant protein either does not bind the nucleotide or is unable to perform the requisite conformational change upon ligand binding.

To examine whether ATP binding per se results in changes in conformation of NBD-512 or if it occurs only after ATP hydrolysis, we also performed these experiments with the NBD-512 C193A mutant. Titration with I^- results in Stern−Volmer plots for NBD-512 C193A that are nearly identical to those obtained for NBD-512 in the absence (f_a = 85%, K_a = 11.7 M^{-1}; Table 2) or presence of Mg-ATP (f_a = 96%, K_{SV} = 9.0 M^{-1}; Table 2). Thus this catalytically deficient mutant undergoes conformational changes in the presence of Mg-ATP just like the native NBD-512. This reiterates the fact that the conformational changes being monitored by us are indeed a result of nucleotide binding rather than due to its hydrolysis.

Fluorescence Quenching with Cs^+. Unlike I^-, when Cs^+ is used as a quencher for the intrinsic fluorescence of NBD-512, it continues to detect a heterogeneous population of Trp in the absence as well as presence of Mg-ATP (Figure 5B). Cs^+ quenches roughly 50% of the total fluorescence emission of NBD-512 and has access to approximately one-third of all Trp residues of NBD-512 in the absence of Mg-ATP (Table 2). When ATP is present, Trp326 must get shielded and Cs^+ should have reduced accessibility to this Trp residue. If no conformational change occurs, this should result in a reduction in the fraction of accessible Trp residues. However, the conformational change accompanying ATP binding results in increased exposure of the other Trp residues of

NBD-512 to the charged quencher resulting in a higher fraction of accessible residues; that is, f_a changes from 32% (p value 0.04) to 47% (p value 0.034).

A mention must also be made of the rather unusually high K_a value for Cs^+ quenching. It seems possible that this is the result of a static quenching complex being formed by the quencher with the protein. We confirmed this by the addition of 150 mM NaCl to disrupt these ionic interactions. As can be seen from Table 2, the higher ionic strength resulted in a lowering of K_a. Of all the Trp residues of NBD-512, since Trp326 has an Asp327 adjacent to it, it is probable that the static quenching complex is formed in its vicinity (discussed later).

For the mutant protein NBD-512 W326A, since Trp326 has been substituted, we do not observe any fluorescence from this residue· Any static quenching complex formed in this pocket will therefore go unnoticed by us. Indeed, as expected, the K_a value (5.0 M^{-1}) for quenching by Cs^+ is low in NBD-512 W326A. The accessibility of the quencher to the Trp residues of this mutant is also low (13%), further suggesting that Cs^+ was originally preferentially "seeing" Trp326 in NBD-512. Additionally, the presence of ATP does not alter the accessibility (15%) of the cationic quencher to the Trp residues of NBD-512 W326A (Table 2, plot not shown). Since at a concentration of 5 mM ATP is hydrolyzed very well by this mutant protein (Figure 3B, bottom panel), it appears that ATP docks but is unable to induce the requisite conformational change in the mutant protein upon binding.

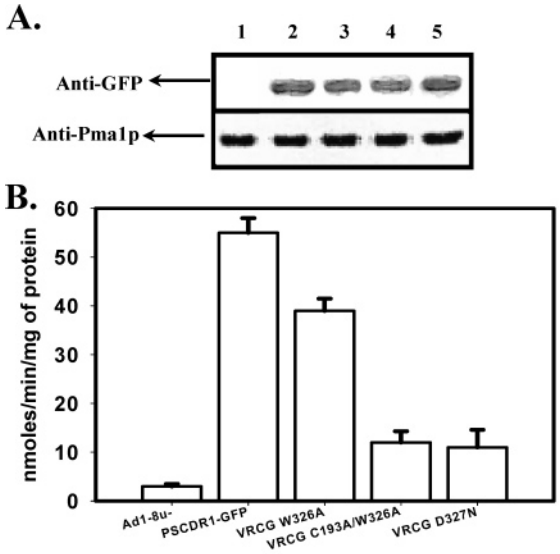
The catalytically affected mutant, NBD-512 C193A, on the other hand, behaves in a manner very similar to native NBD-512 (Table 2, plot not shown). Since ATP binding is not altered in this mutant (*14*) and ATP binding continues to cause conformational change in this protein, this implies that nucleotide binding alone is sufficient for the conformational change that we observe in native NBD-512 as well.

Conserved Asp327 Is Important for ATPase Activity but Not for ATP Binding. Having thus shown that Trp326 is important for ATP binding and the subsequent conformational change, it remained to be ascertained whether the conserved Asp327 adjacent to the unusual Trp326 has any role to play in ATP docking or hydrolysis. The role of this nonvariant Asp in coordination of the metal ion (Mg^{2+}) had been shown earlier to affect the ability of NBDs to bind, hydrolyze, and release nucleotide in other ABC drug transporters (*39*−*43*). Structural data for nonfungal ABC transporters also confirm that the conserved Asp participates in coordination of Mg^{2+} in the enzyme−substrate complex and is significant for ATPase activity of the nucleotide binding domain (*44*−*46*). We constructed two types of mutants, one in which the Asp was replaced by Ala (NBD-512 D327A) and another in which the Asp was replaced with Asn (NBD-512 D327N). We expressed and purified these mutant proteins NBD-512 D327A and NBD-512 D327N as well as the double mutants NBD-512 W326A/D327N and NBD-512 C193A/W326A in a manner similar to that described for NBD-512. The expression levels of the mutant proteins were comparable to that of wild-type NBD-512 (data not shown). We observed that mutating the conserved Asp327 to either Ala (87% reduced activity) or Asn (89% reduced activity) had a similar drastic effect on the enzymatic activity of the protein as was observed in the case of NBD-512 C193A/W326A (data not shown). The NBD-512 D327N mutant also displayed ATP-dependent quenching of intrinsic fluorescence and ATP binding comparable to NBD-512 (K_d = 91 ± 4 μM, Table 1). ATP binding to NBD-512 D327N is Mg^{2+}-dependent and can be abolished by EDTA (data not shown). Thus, contrary to expectations from other conserved nonfungal ABC transporters (*39*−*43*), it appears that Asp327 does not play a role in coordinating Mg^{2+}. Additionally, while both Cys193 and Asp327 are important for the catalytic activity of NBD-512, they are not critical in nucleotide binding by the domain.

Of note, like in the case of NBD-512, ATP binding to the mutant protein resulted in conformational change and enhanced accessibility of charged quenchers to the Trp residues of NBD-512 D327N (Table 2). As can also be seen from Table 2, the K_a value for Cs^+ with NBD-512 D327N is low (6.8 M^{-1}) as compared to that for NBD-512 (K_a = 17.2 M^{-1}). Thus, the removal of the negative charge next to Trp326 brings down the K_a value for the quencher, just as increasing ionic strength by NaCl (150 mM) of the buffer brought down the K_a for NBD-512, reconfirming that the quencher does indeed form a static quenching complex at this residue in NBD-512.

Mutations in Full-Length Cdr1p. To examine the in vivo significance of the uncommon Trp326 and conserved Asp327 in the Walker B domain of the N-terminal NBD (NBD 1) of the full-length protein, we introduced substitution mutations Trp326 to Ala (W326A), Asp327 to Asn (D327N), and the combination mutations C193A/W326A in full-length native Cdr1p. For this, we used a hyperexpression system where Cdr1p (CDR1-GFP) was stably overexpressed from *PDR5* locus in a *Saccharomyces cerevisiae* mutant, AD1-8u$^-$ (*19*). The AD1-8u$^-$ was derived from a *Pdr1-3* mutant strain with a gain-of-function mutation in the transcription factor Pdr1p, resulting in constitutive hyperinduction of the *PDR5* promoter (*18*). The cells expressing these mutant variants of NBDs were designated as VRCG W326A, VRCG C193A/W326A, and VRCG D327N. The stable single-copy integration in our heterologous hyperexpression system was confirmed by Southern hybridization (data not shown). The surface localization of native Cdr1p-GFP in these mutant variants remains unaffected as confirmed by Western blot of the plasma membrane (PM) proteins (Figure 6A). Confocal images further excluded the possibility of mislocalization and altered expression of Cdr1p (CDR1-GFP) due to mutations within NBD1 (data not shown).

Cdr1p W326A Variant Has Higher K_M for ATP Hydrolysis. We performed ATPase assays with the PM of wild-type and mutants cells to check whether mutations in NBD1 in anyway affected the ATPase activity of the full protein. Purified PM fractions from cells expressing native Cdr1p (CDR1-GFP) exhibit a significant increase in ATPase activity as compared to that of host cells (AD1-8u$^-$). This substantial and hence measurable difference in oligomycin-sensitive ATPase activity gave us the opportunity to check the effect of mutations in Walker B of NBD1 in Cdr1p. As depicted in Figure 6B, substitution of Trp326 by Ala in Cdr1p resulted in 30−40% reduction in ATPase activity, similar to that observed for the isolated domain NBD-512 W326A with respect to NBD-512 (Figure 3B, bottom panel). The K_M for ATP hydrolysis in PM preparations expressing native Cdr1p was found to be 0.055 ± 0.004 μM, while that for Cdr1p W326A was 3-fold higher (0.18 ± 0.007 μM). As expected, the V_{max}



C.

MIC_{80} (μg/ml)

	Flu	Aniso	Mico	Cyh
1. AD1-8 u⁻	0.5	0.25	0.00625	0.03125
2. PSCDR1-GFP	64	16	1.0	1.0
3. VRCG W326A	32	16	1.0	0.5
4. VRCG C193A/W326A	1.0	0.5	0.125	0.006
5. VRCG D327N	1.0	0.5	0.125	0.006

FIGURE 6: (A) Expression profile of wild-type and mutant Cdr1 proteins. Plasma membrane of wild-type and mutant proteins were prepared as described earlier (*19*). Immunodetection of proteins was performed by separating the PM proteins (20 μg) from AD1-8u$^-$ (lane 1), PSCDR1-GFP (lane 2), VRCG W326A (lane 3), VRCG C193A/W326A (lane 4), and VRCG D327N (lane 5) on an 8% SDS−PAGE, electroblotted on nitrocellulose membrane and incubated with mouse monoclonal anti-GFP antibody (diluted 1:1000, upper panel) and rabbit polyclonal anti-Pma1p antibody (diluted 1:10 000, lower panel). Proteins were detected by chemiluminescence using an ECL Kit (Amersham). (B) Comparison of ATPase activity of Cdr1p with its mutants. ATPase activity of plasma membrane fraction of cells expressing the wild-type Cdr1p (CDR1-GFP) and its mutant variants was assayed as described previously (*19*), and each reaction was performed in triplicates; the values plotted (with ±SD) represent the average of three independent experiments. The *p*-values for all mutant variants were calculated, and all are statistically significant, VRCG W326A (*p*-value 0.039), VRCG C193A/W326A (*p*-value 0.007), and VRCG D327N (*p*-value 0.007). (C) Drug resistance profiles of the wild-type and mutants determined by minimum inhibitory concentration (MIC) as described earlier (*19*). MIC_{80} test end point was defined as the lowest drug concentration that gave >80% inhibition in growth. (Flu, fluconazole; Aniso, anisomycin; Mico, miconazole; Cyh, cycloheximide.)

values for this activity were not greatly affected (Cdr1p 43.0 ± 2.0 nmol min^{-1} mg protein^{-1}; Cdr1p W326A 60.0 ± 3.5 nmol min^{-1} mg protein^{-1}; LB plots not shown). Thus, similar to our results from isolated domains (NBD-512 and NBD-512 W326A), the mutation of Trp326Ala primarily resulted in an altered affinity for substrate ATP in the mutant protein Cdr1p W326A as compared to native Cdr1p.

On the other hand, the mutation D327N in full-length Cdr1p caused a drastic loss (80−90%) in ATPase activity (Figure 6B). Thus Asp327 to Asn substitution resulted in loss of ATP hydrolysis in a manner similar to that seen in substitution of the uncommon Cys193 by Ala in full-length Cdr1p (*47*).

Cells Expressing Cdr1p W326A Do Not Exhibit Altered Drug Sensitivity. We examined the effect of mutations in the Walker B motif of NBD1 on drug sensitivity of cells expressing full-length Cdr1p. MIC_{80} (minimum inhibitory concentration for 80% inhibition in growth) assays revealed that the host strain (AD1-8u$^-$) is expectedly sensitive to all the drugs when compared to the substantial growth observed in the presence of drugs for the cells expressing native Cdr1p (Figure 6C-table). As compared to the host strain (AD1-8u$^-$), the MIC_{80} for cells expressing wild-type Cdr1p is considerably higher wherein 64 μg/mL of fluconazole, 16 μg/mL of anisomycin, 1.0 μg/mL of miconazole, or 1.0 μg/mL of cycloheximide is required to inhibit 80% growth. Interestingly, cells expressing the mutant Cdr1p W326A also have comparable MIC_{80} values for these drugs (Figure 6C-table). However, cells expressing either Cdr1p D327N or the double mutant Cdr1p C193A/W326A remained hypersensitive to these drugs and accordingly displayed extremely low MIC_{80} values. The results of spot assays also corroborated with the drug sensitivity profile of cells expressing native and mutant Cdr1p proteins (data not shown).

While the double mutation C193A/W326A as well as single mutation D327N led to severe loss in ATPase activity and made cells expressing these mutations sensitive to drugs, the mutation W326A does not dramatically alter the drug sensitivity profile of cells expressing full-length protein. Clearly, the ability of the N-terminal NBD in Cdr1p to hydrolyze ATP correlates well with the drug sensitivity of the cells in which it is expressed, whereas, apparently, its ability to bind ATP does not. However, since the K_M for ATP hydrolysis by the mutant Cdr1p W326A protein is affected (0.18 μM), this must imply that the concentration of ATP in the cell must be sufficient to allow this protein to function, efflux out drugs, and thereby confer drug resistance to the cells.

DISCUSSION

NBDs of most ABC multidrug transporters possess highly conserved amino acid sequences and are therefore expected to be quite similar in structure, implying thereby that they may also be mechanistically very similar (*1*, *2*). Our present as well as previous results show that those in the fungal world, however, are probably not completely analogous to their mammalian counterparts (*12*, *13*). For example, we have recently demonstrated that the uncommon but conserved Cys193 in the Walker A motif of the N-terminal NBD of Cdr1p, is important for ATP hydrolysis by the protein (*14*, *15*). Thus, if Cys193 is replaced by Ala, either in the isolated domain NBD-512 or in the full length Cdr1p protein, it results in severely impaired ATPase activity without any loss in nucleotide binding. Of note, Cys193 in the Walker A motif of NBD1 is a feature common to all fungal ABC transporters as well but is replaced by a well-conserved Lys at an equivalent position in other ABC transporters (*12*, *13*).

We now demonstrate that the unique positioning of an aromatic tryptophan residue preceding the well-conserved Asp327 in the Walker B motif of NBD1 is another

characteristic feature of Cdr1p and other fungal ABC transporters (*12*, *13*). Notably, other ABC transporters of bacterial and mammalian origin have hydrophobic amino acids preceding the Asp in place of Trp (*1*, *39*−*41*), but any role in ATP binding for these residues has not been proposed so far. We show in this study that ATP binding by NBD-512 requires the presence of Trp326 in the Walker B motif of the binding pocket. We observed that Mg^{2+}-dependent ATP binding resulted in a significant and specific intrinsic tryptophan fluorescence quenching of the isolated domain NBD-512. Chemical modification and site-directed mutagenesis of the five Trp residues in NBD-512 further confirmed that the uncommon Trp326 is important for nucleotide binding.

We have also observed that Mg-dependent ATP binding to the native NBD-512 induces a conformational change in the molecule that leads to increased accessibility of its other Trp residues to charged quenchers (Table 2). Furthermore, we show that this conformational change is specifically a consequence of ATP docking rather than its hydrolysis in the native NBD-512. This is supported by the fact that accessibility of charged quenchers to Trp residues in catalysis-deficient mutants (NBD-512 C193A and NBD-512 D327N that are unaffected in ATP binding) resembles that for native NBD-512 protein. This is not surprising, since it is now very well-accepted in the case of ABC proteins that nucleotide binding alone is capable of inducing significant conformational change in the NBD (reviewed in ref *48*). It appears however that Mg-ATP is unable to enforce this conformational change in NBD-512 W326A. Thus, the NBD-512 W326A mutant protein does not have increased accessibility to charged quenchers in the presence of Mg-ATP as compared to that in the absence of Mg-ATP.

It is probable that the Trp326 residue is critical in maintaining the integrity of the ATP binding pocket due to either strictly steric considerations, given that it has an extremely bulky side chain, or direct coordination with the cation. That such subtle changes are likely to have occurred in the binding pocket due to the substitution of Trp326 is supported by the fact that charged quenchers have much less accessibility to Trp residues in NBD-512 W326A as compared to those in wild-type NBD-512 (Table 2). It is possible that these subtle changes alone are sufficient to reduce the interaction of ATP within the nucleotide-binding pocket and are responsible for the loss in conformational change that must accompany nucleotide binding to the domain.

However, another equally likely explanation exists. We note that the changes in the binding pocket are not drastic. The fact that the fluorescence spectrum undergoes a shift neither in its emission maximum nor in its shape as a result of this mutation indicates that the overall conformation of the domain is not dramatically altered by the mutation. In all likelihood, the orientation of the catalytic residues in the hydrolysis pocket remains largely unchanged also. This contention is supported by the fact that NBD-512 W326A continues to hydrolyze ATP (Figure 3B, bottom panel), albeit with a significantly higher K_M value. We propose therefore that two events contribute to the strength of the affinity that the native domain has for its ligand. The first involves a specific recognition of the ligand by the binding pocket. The second involves a conformational change in the domain, an induced-fit that greatly increases the strength of the interaction once the ligand has docked into the binding site. It is possible that the substitution of Trp326Ala has a larger effect upon this latter step. So, although the binding pocket recognizes the ligand, the subsequent steps that would increase the strength of the interaction do not occur. Consequently, the K_M for ATP binding by the mutant NBD-512 W326A is higher. The higher V_{max} value for the mutant variant NBD-512 W326A is perhaps indicative of this very fact. Poor docking of the substrate due to the inability of the domain to affect the subsequent conformational change results in a greater ease of leaving for the product (ADP and phosphate) as well. Such a role in ATP binding for the hydrophobic amino acid situated in the Walker B motif of the nucleotide-binding domain is unusual. We to point out, however, that a conserved aromatic residue upstream of the Walker A motif in NBDs of several other nonfungal ABC transporters has been shown to be important for ATP binding (*25*, *44*, *49*).

Additionally, contrary to the role of the well-conserved Asp (equivalent to Asp327 of NBD-512) in Mg^{2+} coordination in other NBDs (*39*−*43*), we observed that the mutant NBD-512 D327N not only exhibited Mg^{2+}-dependent ATP binding but also underwent subsequent conformational changes similar to NBD-512 (data not shown). This would imply that Asp327 does not play its traditional role of Mg^{2+} coordination and ATP binding in NBD-512. Nevertheless, Asp327 appears to be important for ATP hydrolysis since the mutant NBD-512 D327N exhibited severely impaired ATPase activity as compared to NBD-512 and behaves analogous to the catalytically deficient mutant NBD-512 C193A (*14*, *15*). The fact that substitution of either Cys193 or Asp327 results in a mutant full-length Cdr1p protein that is incapable of hydrolyzing ATP further supports their role in catalysis. As a consequence of such substitutions in the full-length Cdr1p protein, cells expressing these mutant proteins (Cdr1p C193A or Cdr1p D327N) are extremely sensitive to drugs, apparently because these mutant versions of Cdr1p are incapable of hydrolyzing ATP and thus cannot power drug efflux.

We also point out that the full-length Cdr1p and its mutant variant Cdr1p W326A are likely to be functional in situ at far lower ATP concentrations as compared to the corresponding isolated domains, which also explains why they continue to function within the cell and confer drug resistance. A careful comparison of K_M and V_{max} values shows that in absolute terms the ATPase activities from full-length proteins are much higher than those exhibited by the isolated domains. Thus full-length Cdr1p and its mutant Cdr1p W326A have much lower K_M values for ATP hydrolysis as compared to their respective isolated domains. Thus, if the ATP concentration within the cell is high enough to overcome the higher K_M requirement for ATP hydrolysis, the NBDs of Cdr1p W326A will continue to bind and hydrolyze ATP and thereby confer drug resistance to cells expressing this protein. The recent paper of Zhao and Chang (*49*) reports a very similar effect when Trp653 or Tyr1302, conserved aromatic residues upstream of the Walker A motif in the two NBDs of MRP1, were substituted by Cys. Neither the single nor double mutations in full-length MRP1 altered leukotriene C4 transport, although ATP binding itself was weaker. Thus, in general, it would seem that merely altering ATP binding capacity does not alter the ability of the ABC

transporter to hydrolyze ATP and mediate drug transport within the cell. It would however be very interesting to study the effect of this impaired binding in cells that are ATP-starved.

It is important to point out that the presence of Cys in NBD1 of fungal ABC transporters in place of the well-conserved Lys in other nonfungal homologues had led to the thinking that NBD1 of these transporters are probably nonfunctional. However, our recent report (*47*) as well as the present study amply demonstrates that NBD1 of Cdr1p is indeed functional and substitution of either Cys193 or Asp327 can make this ABC transporter nonfunctional. If the two NBDs were functionally equivalent, as in the case of histidine permease (*50*), inactivating one of the domains would have resulted in a 50% reduction of the function of the protein rather than a complete loss in drug resistance that we observe. On the other hand, if binding and release of ATP rather than its hydrolysis was sufficient for drug transport as has been hypothesized for MRP1 (*49*), then the mutation of Cys193 or Asp327 in the full-length protein Cdr1p should not have resulted in cells that were extremely drug-sensitive. Apparently, ATP hydrolysis by NBD1 of Cdr1p is critical for drug transport by the full-length protein. Since no mutations were done in the C-terminal NBD2, one expects it to be unaltered in its function. Yet, NBD2 is unable to compensate for the loss of ATP hydrolysis in NBD1. Thus, the two NBDs of Cdr1p, and probably of other fungal transporters as well, are functionally asymmetric.

Taken together, our work provides the first biochemical and biophysical proof that it is possible to separate the binding and catalytic events of NBD1 of the ABC transporter Cdr1p by the construction of a binding-deficient mutant (NBD-512 W326A) as opposed to a catalytically defunct one (NBD-512 D327N or NBD-512 C193A). The ATP binding event, for which Trp326 is important, functions independently of the hydrolysis step for which Cys193 in the Walker A motif or Asp327 in the Walker B motif are critical. The fact that the role of these residues could also be demonstrated in the full-length Cdr1p protein excludes any possibility of artifactual effects and points to basic mechanistic differences between fungal and mammalian ABC transporters. The elucidation of mechanistic differences in ligand binding and hydrolysis between fungal and nonfungal ABC transporters is under active investigation. Given that some of these ABC transporters play a major role in antifungal resistance, these mechanistic differences between host and pathogen will probably have considerable therapeutic implications.

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REFERENCES

1. Holland, I. B., Cole, S. P. C., Kuchler, K., and Higgins, C. F. (2003) *ABC Proteins from Bacteria to Man*, Academic Press, San Diego, CA.
2. Walmsley, M. B., Mckeegan, K. S., and Walmsley, A. R. (2003) Structure and function of efflux pumps that confer resistance to drugs, *Biochem. J. 376*, 313−338.
3. Calderone, R. A. (2002) *Candida and Candidiasis*, 1st ed., ASM Press, Washington, DC.
4. Prasad, R., Panwar, S. L., and Smriti. (2002) Drug resistance in yeasts—an emerging scenario, *Adv. Microb. Physiol. 46*, 155−201.
5. White, T. C. (1997) Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus, *Antimicrob. Agents Chemother. 41*, 1482−1487.
6. Hernaz, M., Gill, C., Pla, J., and Nomela, C. (1998) Induced expression of *Candida albicans* multidrug resistance gene CDR1 in response to fluconazole and other antifungals, *Yeast 14*, 517−526.
7. Rex, J. H., Rinaldi, M. G., and Pfaller, M. A. (1995) Resistance of candida species to fluoconazole, *Antimicrob. Agents Chemother. 39*, 1−8.
8. Sanglard, D., Ischer, F., Monod, M., and Bille, J. (1997) Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene, *Microbiology 143*, 405−416.
9. Albertson, G. D., Niimi, M., and Cannon, R. D. (1996) Multiple efflux mechanisms are involved in *Candida albicans* fluoconazole resistance, *Antimicrob. Agents Chemother. 40*, 2835−2841.
10. Sanglard, D., Kuchler, K., Ischer, F., Pagani, J. L., Monod, M., and Bille, J. (1995) Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters, *Antimicrob. Agents Chemother. 39*, 2378−2386.
11. Krishnamurthy, S., Gupta, V., Prasad, R., Panwar, S. L., and Prasad, R. (1998) Expression of *CDR1*, a multidrug resistance gene of *Candida albicans*: in vitro transcriptional activation by heat shock, drugs and human steroid, *FEMS Microbiol. Lett. 160*, 191−197.
12. Prasad, R., Gupta, N., and Gaur, M. (2004) Molecular basis of antifungal resistance, in *Pathogenic Fungi*, pp 357−414, Caister Academic Press, Norfolk, U.K.
13. Decottignies, A., Goffeau, A. (1997) Complete inventory of the yeast ABC proteins, *Nat. Genet. 15*, 137−145.
14. Jha, S., Karnani, N., Dhar, S. K., Mukhopadhayay, K., Shukla, S., Saini, P., Mukhopadhayay, G., Prasad, R. (2003) Purification and characterization of the N-terminal nucleotide binding domain of an ABC drug transporter of *Candida albicans*: uncommon cysteine 193 of Walker A is critical for ATP hydrolysis, *Biochemistry 42*, 10822−10832.
15. Jha, S., Karnani, N., Lynn, A. M., Prasad, R. (2003) Covalent modification of cysteine 193 impairs ATPase function of nucleotide-binding domain of a Candida drug efflux pump, *Biochem. Biophys. Res. Commun. 310*, 869−875.
16. Spande, T. F., and Witkop, B. (1967) Determination of tryptophan content of proteins with *N*-bromosuccinimide, *Methods Enzymol. 11*, 498−506.
17. Lakowicz, J. R. (1999) *Principles of Fluorescence Spectroscopy*, 2nd ed., Plenum Publishers, New York.
18. Nakamura, K., Niimi, M., Niimi, K., Holmes, A. R., Yates, J. E., Decottignies, A., Monk, B. C., Goffeau, A., and Cannon, R. D. (2002) Functional expression of *Candida albicans* drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transporters, *Antimicrob. Agents Chemother. 45*, 3366−3374.
19. Shukla, S., Saini, P., Smriti, Jha, S., Ambudkar, S. V., and Prasad, R. (2003) Functional characterization of *Candida albicans* ABC transporter Cdr1p, *Eukaryotic Cell 2*, 1361−1375.
20. Biswas-Fiss, E. E. (2003) Functional analysis of genetic mutations in nucelotide binding domain 2 of human retina specific ABC transporter, *Biochemistry 42*, 10683−10696.
21. Zhou, T., and Rosen, B. P. (1997) Tryptophan fluorescence reports nucleotide-induced conformational changes in a domain of the ArsA ATPase, *J. Biol. Chem. 272*, 19731−19737.
22. Dayan, G., Baubichon-Cortay, H., Jaul, J.-M., Cortay, J.-C., Deleage, G., and Di Pietro, A. (1996) Recombinant N-terminal nucleotide-binding domain from mouse P-glycoprotein: overexpression, purification, and role of cysteine 430, *J. Biol. Chem. 271*, 11652−11658.
23. Baubichon-Cortay, H., Baggetto, L. G., Dayan, G., and Pietro, A. D. (1994) Over-expression and purification of the carboxyl-terminal nucleotide-binding domain from mouse P-glycoprotein: strategic location of a tryptophan residue, *J. Biol. Chem. 269*, 22983−22989.
24. Schneider, E., Wilken, S., Schmid, R. (1994) Nucleotide-induced conformational changes of MalK, a bacterial ATP binding cassette transporter protein, *J. Biol. Chem. 32*, 20456−20461.
25. Ramaen, O., Masscheleyn, S., Duffieux, F., Pamlard, O., Oberkampf, M., Lallemand, J. Y., Stoven, V., and Jacquet, E. (2003) Biochemical characterization and NMR studies of nucleotide

binding domain 1 of multidrug-resistance-associated protein 1: evidence for interaction between ATP and Trp, *Biochem. J. 376*, 749−756.
26. Eriksson, S., Norden, B., Morimatsu, K., Horii, T., Takahaski, M. (1993) Role of tyrosine residue 264 of RecA for the binding of cofactor and DNA, *J. Biol. Chem. 268*, 1811−1816.
27. Liu, R., Sharom, F. J. (1997) Fluorescence studies on the nucleotide binding domains of the P-glycoprotein multidrug transporter, *Biochemistry 36*, 2836−2843.
28. Marmarosh, N., Gimi, K., Urbatsch, I. L., Gros, P., and Senior, A. E. (1999) Large scale purification of detergent soluble P-glycoprotein from *Pichia pastoris* cells and characterization of nucleotide binding domain properties of wild type, Walker A, and Walker B mutant proteins, *J. Biol. Chem. 274*, 34711−34718.
29. Liu, R., Siemiarczuk, A., and Sharom, F. J. (2000) Intrinsic fluorescence of the P-glycoprotein multidrug transporter: sensitivity of tryptophan residues to binding of drugs and nucleotides, *Biochemistry 39*, 14927−14938.
30. Lansky, Z., Kubala, M., Ettrich, R., Kuty, M., Plasek, J., Teisinger, J., Schoner, W., and Amler, E. (2004) The hydrogen bonds between Arg423 and Glu472 and other key residues, Asp443, Ser477, and Pro489, are responsible for the formation and a different positioning of TNP-ATP and ATP within the nucleotide-binding site of Na (+)/K (+)-ATPase, *Biochemistry 43*, 8303−8311.
31. Kubala, M., Plasek, J., and Amler, E. (2004) Protein modeling combined with spectroscopic techniques: an attractive quick alternative to obtain structural information, *Physiol. Res. 53*, 109−113.
32. Mourez, M., Hofnung, M., and Dassa, E. (1997) Subunit interactions in ABC transporters: a conserved sequence of periplasmic permease defines an important site of interaction with the ATPase subunit, *EMBO J. 16*, 3066−3077.
33. Wang, G., Pincheira, R., Zhang, J. T. (1998) Dissection of drug-binding-induced conformational changes in P-glycoprotein, *Eur. J. Biochem. 255*, 383−390.
34. Martin, C., Berridge, G., Mistry, P., Higgins, C., Charlton, P., and Callaghan, R., (2000) Drug binding sites on P-glycoprotein are altered by ATP binding prior to nucleotide hydrolysis, *Biochemistry 39*, 11901−11906.
35. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2003) Drug binding in human P-glycoprotein causes conformation changes in both nucleotide-binding domains, *J. Biol. Chem. 278*, 1575−1578.
36. Eftnik, M. R., and Ghiron, C. A. (1976) Exposure of tryptophanyl residues in proteins. Quantitative determination by fluorescence quenching studies, *Biochemistry 15*, 672−680.
37. Eftnik, M. R., and Ghiron, C. A. (1977) Exposure of tryptophanyl residues and protein dynamics, *Biochemistry 16*, 5546−5551.
38. Eftnik, M. R., and Ghiron, C. A. (1981) Fluorescence quenching studies with proteins, *Anal. Biochem. 114*, 199−227.
39. Urbatsch, I. L., Julien, M., Carrier, I., Rousseau, M. E., Cayrol, R., and Gros, P. (2000) Mutational analysis of conserved carboxylate residues in the nucleotide binding sites of P-glycoprotein, *Biochemistry 39*, 14138−14149.
40. Sauna, Z. E., Muller, M., Peng, X. H., and Ambudkar, S. V. (2002) Importance of the conserved Walker B glutamate residues, 556 and 1201, for the completion of the catalytic cycle of ATP hydrolysis by human P-glycoprotein (ABCB1), *Biochemistry 41*, 13989−14000.
41. Carrier, I., Julien, M., and Gros, P. (2003) Analysis of catalytic carboxylate mutants E552Q and E1197Q suggest asymmetric ATP hydrolysis by the two nucleotide-binding domains of P-glycoprotein, *Biochemistry 42*, 12875−12885.
42. Urbatsch, I. L., Tyndall, G. A., Tombline, G., and Senior, A. E. (2003) P-glycoprotein catalytic mechanism: studies of the ADP-vanadate inhibited state, *J. Biol. Chem. 278*, 23171−23179.
43. Payen, L. F., Gao, M., Westlake, C. J., Cole, S. P., and Deeley, R. G. (2003) Role of carboxylate residues adjacent to the conserved core Walker B motifs in the catalytic cycle of multidrug resistance protein 1 (ABCC1), *J. Biol. Chem. 278*, 38537−38547.
44. Hung, L. W., Wang, I. X., Nikaido, K., Liu, P. Q., Ames, G. F., and Kim, S. H. (1998) Crystal structure of the ATP-binding subunit of an ABC transporter, *Nature 396*, 703−707.
45. Hopfner, K. P., Karcher, A., Shin, D. S., Craige, L., Arthur, L. M., Carney, J. P., and Tainer, J. A. (2000) Structural biology of Rad50 ATPase: ATP driven conformation control in DNA double strand break repair and the ABC-ATPase superfamily, *Cell 101*, 789−800.
46. Verdon, G., Alberer, S. V., Dijkstra, B. W., Driessen, A. J., and Thunnissen, A. M. (2003) Crystal structure of ATPase subunit of ABC transporter of *Sulfolobus solfataricus:* nucleotide free and nucleotide bound conformations, *J. Mol. Biol. 330*, 343−358.
47. Jha, S., Dabas, N., Karnani, N., and Prasad, R. (2004) ABC multidrug transporter Cdr1p of *Candida albicans* has divergent nucleotide-binding domain, which display functional asymmetry, *FEMS Yeast Res. 5*, 63−72.
48. Linton. K. J., and Higgins. C. F. (2004) The ATP switch model for ABC transporters, *Nat. Struct. Mol. Biol. 11*, 918−926.
49. Zhao, Q., and Chang, X. B. (2004) Mutation of the aromatic amino acid interacting with adenine moiety of ATP to a polar residue alters the properties of MRP,. *J. Biol. Chem. 279*, 48505−48512.
50. Nikaido, K., and Ames, F.-L. G. (1999) One intact ATP binding subunit is sufficient to support ATP hydrolysis and translocation in an ABC transporter, the histidine permease, *J. Biol. Chem. 274*, 26727−26735.
51. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.
52. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle, *Anal. Biochem. 72*, 248−254.

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