Investigation of the Role of Glutamine-471 and Glutamine-1114 in the Two Catalytic Sites of P-Glycoprotein†

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ABSTRACT: P-glycoprotein, also known as multidrug resistance protein, pumps drugs out of cells using ATP hydrolysis as the energy source. Glutamine-471 and the corresponding glutamine-1114 in the two catalytic sites of P-glycoprotein are conserved in ABC transporters. X-ray structures show that they lie close to the bound nucleotide. Proposed functional roles are (1) activation of the attacking water for ATP hydrolysis, (2) coordination of the essential Mg^{2+} cofactor in Mg nucleotide, and (3) signal communication between catalytic site reaction chemistry and drug-binding sites. We made mutations Q471A, Q471E, Q1114A, and Q1114E in mouse MDR3 P-glycoprotein. Pure mutant and wild-type proteins were prepared and subjected to enzymatic and biochemical characterization. We conclude from the results that the primary role of this glutamine residue is in interdomain signal communication. Coordination of the Mg^{2+} cofactor is not a critical functional role, neither is activation of the attacking water molecule, although an auxiliary role in positioning the water cannot be ruled out. We found that equivalent mutations (Ala or Glu) in either of the two P-glycoprotein catalytic sites produced the same effects, implying functional symmetry of the two sites.

P-glycoprotein (Pgp)[1] confers a multidrug resistance phenotype on cells through its ability to exclude a wide variety of hydrophobic drugs and other compounds. Included among these are anticancer chemotherapeutic drugs and anti-AIDS drugs, and so there is high interest in resolving the structure and understanding the mechanism of action of Pgp in order to learn how to disable it or otherwise circumvent its effects (*1*−*5*). In the domain structure, Pgp is typical of the ABC transporter superfamily (*6*), consisting of two transmembrane domains (*7, 8*) and two nucleotide binding sites (*9*−*11*) contained within a linear sequence of about 1280 amino acids. It achieves lowered intracellular drug concentration by hydrolyzing ATP (*12*−*14*) and acting as an ATP-driven drug efflux pump. Both of the nucleotide sites have been shown to be catalytic sites capable of hydrolyzing ATP, and they act in concert during steady-state catalysis (*15*−*17*). There is a clear requirement for two intact nucleotide sites in order for even single turnover of ATPase to occur (*18*−*20*). The drug binding and transport sites are known to be formed by transmembrane domains (*21*−*25*), located at a considerable distance from the nucleotide sites. A model involving alternating site catalysis, and linkage of drug transport to formation and collapse of the catalytic transition state, was presented by our laboratory (*26*). It has gained general support and has been further elaborated recently (*27*−*30*).

While no high-resolution structure of Pgp is available, the X-ray structures of the nucleotide-binding domains of two other ABC transporters have been resolved, namely, HisP (*31*) and RbsA N-terminal nucleotide site (C. S. Stauffacher, personal communication). The presence of the Walker A, Walker B, and "LSGGQ" signature sequences in all three proteins argues strongly that Pgp catalytic sites resemble those of HisP and RbsA. Further evidence that this is the case comes from photoaffinity labeling of Pgp by 8-azido-ATP. It was found that the homologous Tyr residue (Tyr-397 and Tyr-1040[2]) was labeled in both Pgp catalytic sites (*32*). This Tyr corresponds in sequence to the aromatic residue, Tyr-16 or Phe-14, respectively, which is stacked against the adenine ring of the bound nucleotide in HisP (*31*) or RbsA (C. S. Stauffacher, personal communication) nucleotide-binding sites. Together with the design of a large-scale procedure for purification of mutant Pgp (*33*), the X-ray structures open up the possibility of studying Pgp catalytic sites at the molecular level.

One residue of immediate interest in Pgp is Gln-471 of the N-terminal ATP-binding site and the corresponding Gln-1114 of the C-terminal site. Notably, this Gln residue is conserved in ABC transporters. In both HisP and RbsA X-ray structures, it lies very close to the bound nucleotide. In HisP, where the bound nucleotide is ATP, the NE2 atom of the corresponding Gln-100 lies 2.88 Å from water-437, which is thought to be the "attacking" water, and Gln-100 was therefore considered as a possible activating residue for hydrolysis (*31*). Mg^{2+} is not present in this structure, but water- 407 was thought to occupy the location of Mg^{2+}, and the NE2 atom of Gln-100 lies 3.96 Å from water-407. In the RbsA structure, where the MgADP product is the bound nucleotide, the corresponding Gln-86 OE1 atom is very close to the Mg^{2+}, with which it might form a direct coordination

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[1] Abbreviations: Pgp, P-glycoprotein; DM, *n*-dodecyl *β*-D-maltoside; Vi, orthovanadate; AlFx, fluoroaluminate; BeFx, beryllium fluoride; TPP^+, tetraphenylphosphonium.

[2] Here we use residue numbers corresponding to the mouse MDR3 Pgp sequence.

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ligand. Thus two separate functions may be proposed, namely, direct interaction with the attacking water or with the Mg^{2+} cofactor, and both might be operative, depending on the stage of catalysis; a third function can quite reasonably be added, namely, signal communication between the chemistry occurring at the catalytic site and coupling to drug transport.

In this paper we investigate the possible role(s) of Gln-471 and Gln-1114 in Pgp using a mutagenic analysis. We constructed mutations Q471A and -E and Q1114A and -E in mouse MDR3 Pgp,[3] expressed the mutant proteins in *Pichia pastoris*, purified the proteins to homogeneity, and subjected them to a range of enzymatic and other characterizations.

EXPERIMENTAL PROCEDURES

Introduction of Q471A, Q471E, Q1114A, and Q1114E Mutations into Mouse mdr3 cDNA. Site-directed mutagenesis used the commercially available altered sites II method from Promega. For mutations in the N-terminal ATP-binding site, an *Eco*RI−*Eco*RI fragment of mouse *mdr3* cDNA (bp −13 to 2248) was excised from pDR16 (*34*) and ligated into pAlter-1 to create pAlt-*mdr3*N, which was the template for mutagenesis. To facilitate subsequent manipulations, this template also contained engineered *Nru*I (position 1348) and *Sal*I (position 1908) restriction sites. The mutagenic oligonucleotide for the Q471E mutation was GGTGTGGTGAGTGAGGAGCCTGTGCTGTTT, in which the underlined GAG (Glu) replaced CAG (Gln) and the underlined G introduced a *Bse*RI site. For mutations in the C-terminal ATP-binding site, an *Eco*RI−*Age*I fragment (bp 2248−4007) of mouse *mdr3* cDNA was excised from pVT-*mdr3.5* (*19*), the *Age*I site was blunt ended with T4 DNA polymerase, and the fragment was ligated into pAlter-1, which had been cut with *Eco*RI and *Sal*I and blunt ended at the *Sal*I site, to create pAlt-*mdr3.5*C. A *Psh*AI site was introduced at position 3542, creating pAlt-*mdr3.6*C, which was the template for mutagenesis. To facilitate subsequent manipulations, this template also contained engineered *Nru*I (position 2872), *Sal*I (position 2631), *Spe*I (position 3067), *Sna*BI (position 3980), and *Age*I (position 4007) restriction sites. The mutagenic oligonucleotide for Q1114A mutation was GGCATTGTGTCCGCGGAGCCCATTCTC, where the underlined bases replace Gln (CAA) by Ala (GCG) and introduce a new *Sac*II site. The mutagenic oligonucleotide for Q1114E mutation was GGCATTGTGTCCGAGGAGCCCATTCT, where the underlined bases GAG replace Gln (CAA) by Glu and introduce a new *Bse*RI site. The Q471A mutation was obtained as follows. The template was an *Eco*RI−*Eco*RI fragment (bp −13 to 2248) from *mdr3* cDNA ligated in M13mp18 (*19*), and the mutagenic antisense oligonucleotide was CACAGGTTCCZCACTCACCACAC, in which the mutagenic CZ change Gln (CAG) to Ala (GCG) or Glu (GAG). Only Ala mutants were obtained. Mutagenesis in this case was by the T7-GEN method (USB Corp.). In all cases the presence of the correct mutation and lack of any undesired mutations in the fragment to be transferred were checked by DNA sequencing.

[3] Mouse MDR3 Pgp is 87% identical in amino acid sequence to human MDR1 Pgp. There are no significant differences in functional behavior between the two proteins.

Expression of Mutations in P. pastoris. Expression of mutations utilized the InVitrogen *P. pastoris* expression system (license no. 272749). Mutations were transferred into plasmid pHIL-*mdr3.5*-His6 (ref *19*; Q471A and -E) or pHIL-*mdr3.6*-His6 (same as pHIL-*mdr3.5*-His6, ref *19*, except it has an additional *Psh*AI site at bp 3542, used for Q1114A and -E) using the following restriction fragments: Q471A and Q471E, *Bgl*II−*Sma*I; Q1114A and Q1114E, *Spe*I−*Psh*AI. Successful transfer was checked by restriction digestion and/or DNA sequencing. The new pHIL plasmids were carefully checked using a combination of *Afl*II, *Bgl*II, *Sma*I, and *Sna*BI to ensure that they gave fragments of the correct size; then they were digested with *Not*I, and the linearized DNA (containing the whole *mdr3* sequence on a 9.3 kb fragment) was transformed into *P. pastoris* strain GS115 by the LiCl technique (InVitrogen guidelines). His^+ transformants that showed slow growth when patched on methanol-containing plates (Mut^S) were isolated. These isolates have undergone homologous recombination of the *mdr3* sequence behind the AOX1 promoter. Expression of MDR3 protein was assayed using the "rapid membranes preparation" and Western blotting as previously descibed (*33*). Strains that showed good expression of Pgp were saved as glycerol stocks. Around 90% of His^+, Mut^S recombinants expressed Pgp. The pHIL-*mdr3.5*-His6 and pHIL-*mdr3.6*-His6 plasmids contain the full-length mouse MDR3 Pgp sequence with an additional 6 His-tag at the C-terminal end. Thus all mutant proteins contained the His-tag.

Media and Growth of P. pastoris Cells. Media and growth of cells in 2 L flasks for purification of Pgp were as described previously (*33*) with the following modifications. After initial growth in MGY medium, cells were centrifuged and resuspended in MM medium containing 0.5% v/v methanol, at A_{600} of 3.0−4.0 instead of 2.0 as previously. Further additions of methanol were 1% v/v at 24 and 48 h to increase cell yield.

Purification of Pgp. Cells were harvested and resuspended as described (*33*) and disrupted in 100 g batches in a Bead-Beater (Biospec Inc., Model 1107900) using 0.5 mm diameter glass beads (catalog no. 11079-105). (Previously a French press was used.) Crude microsomes were prepared from disrupted cells as previously described (*33*). Solubilization and purification of Pgp were as previously described (*33*), using DM for solubilization, followed by Ni-affinity chromatography, DEAE-cellulose ion exchange, and concentration by pressure filtration. After the solubilization step, all buffers contained 20% v/v glycerol (instead of 30% in ref *33*) to speed chromatography and concentration.

Activation of Pgp ATPase with DTT and Lipid. This was done as described in ref *33*. Briefly, Pgp was incubated with 8 mM DTT and 1% w/v *Escherichia coli* lipids (Avanti, acetone/ether preparation) (final ratio of lipid/protein = 100/1) for 20 min at room temperature, followed by sonication for 30 s at 4 °C in a bath sonicator. Where labeling with or trapping of radioactive nucleotide was being assayed, the ratio of lipid/protein was reduced to 2/1 (which is sufficient for maximal activation of ATPase; ref 33) to reduce nonspecific binding of nucleotide.

Assays of ATPase Activity. Aliquots of 2−10 μL of activated Pgp (above) were added to 50 μL of 50 mM Tris-HCl, pH 7.5, 10 mM NaATP, and 10 mM $MgCl_2$ and incubated at 37 °C. Reactions were stopped at appropriate

times by addition of 1 mL of ice-cold 20 mM H_2SO_4, and P_i release was estimated by the method of Van Veldhoven and Mannaerts (*35*). All reactions were linear with time, and ≤10% of the ATP was hydrolyzed. Verapamil was added as a solution in dimethyl sulfoxide, keeping solvent ≤2% v/v. For determination of K_M values, MgATP at varied concentrations was added with excess 2 mM $MgCl_2$. Mg^{2+} dependence of ATP hydrolysis was measured using 10 mM NaATP and 150 μM verapamil in 50 mM Tris-HCl, pH 7.5, with $MgCl_2$ at varied concentrations. The pH dependence of ATPase activity was done in the presence of 150 μM verapamil, using Tris−succinate buffer as in refs *13* and *14*. Competitive inhibition by MgADP was assayed in the presence of verapamil, at varied concentrations of MgATP and added MgADP. Inhibition of ATPase by vanadate (Vi), beryllium fluoride (BeFx), or fluoroaluminate (AlFx) was assayed using MgATP (10 mM) and 150 μM verapamil for 60 min at 37 °C. The Vi concentration was varied (see Results). For BeFx and AlFx a constant concentration of 5 mM NaF was used with 0.2 mM $BeSO_4$ or 1 mM $AlCl_3$. Orthovanadate solutions (100 mM) were prepared from Na_3VO_4 (Fisher Scientific) at pH 10 and boiled for 2 min before each use to break down polymeric species.

Centrifuge Column Elution of Pgp. One milliliter centrifuge columns of Sephadex G-50 in disposable syringes were equilibrated with 50 mM Tris-HCl, pH 7.5, and 0.001% DM at 20 °C. After a prespin as described (*36*), Pgp (2.5−20 μg in 100 μL volume) was loaded on the column and eluted by a second spin at 20 °C. Recovery of Pgp in the eluates was 80−95% as judged by ATPase and protein assays.

Photoaffinity Labeling with 8-Azido-[α-^{32}P]ATP. Activated Pgp (2 μg) was incubated in 20 μL containing 2.5 μM 8-azido-[α^{32}P]ATP, 150 μM verapamil, and 50 mM Tris-HCl, pH 7.5, in the presence of EDTA (1 mM) or $MgCl_2$ as indicated, for 5 min on ice. The final DTT concentration was 1 mM. This was followed by UV irradiation for 5 min on ice (UVG-11 Minerallight, 254 nm, 5.5 mW/cm^2, placed directly above the samples). Photolabeled samples were mixed with SDS buffer, and the whole sample was run on 10% SDS gels, stained with Coomassie Blue, and subjected to autoradiography.

Vi-Induced Trapping of Nucleotide with 8-Azido-[α-^{32}P]-ATP. Activated Pgp (3.5 μg) was incubated with 100 μM 8-azido-[α-^{32}P]ATP, 200 μM Vi, 150 μM verapamil, and 50 mM Tris-HCl, pH 7.5, in a total volume of 50 μL at 37 °C for 20 min. EDTA or $MgCl_2$ was included at 3 mM concentration. Incubations were started by addition of 8-azido-[α-^{32}P]-ATP and stopped by transfer to ice. A total of 50 μL of ice-cold 50 mM Tris-HCl, pH 7.5, was added to bring the volume to 100 μL, and unbound ligands were promptly removed by passage though a centrifuge column (above). Samples were irradiated on ice with UV for 5 min as above. Ten microliters of SDS buffer was added, and the samples were incubated for 10 min at 37 °C, then concentrated to 15 μL volume in a SpeedVac at 43 °C, and run on SDS gels.

Routine Procedures. Protein concentrations were determined by the bicinchoninic acid method in the presence of 1% SDS using bovine serum albumin as a standard (*13*). SDS−PAGE was done using the Mini-PROTEAN II gel and Electrotransfer system (Bio-Rad). Samples (2 volumes) were dissolved in 1 volume of 5% (w/v) SDS, 25% (v/v) glycerol, 0.125 M Tris-HCl, pH 6.8, 0.01% pyronin Y, and 160 mM DTT for 20 min at 37 °C and then run on 10% polyacrylamide gels. For immunodetection of Pgp, mouse monoclonal antibody C219 (Signet Laboratories Inc.) was used with the ECL detection system (Amersham). For autoradiography, SDS gels were stained with Coomassie Blue, dried, and exposed overnight at −80 °C to Kodak BioMax films with intensifying screens. To quantitate the amount of radioactivity in the Pgp bands, a PhosphoImager (Storm 860, Molecular Dynamics) and ImageQuant 5.0 software (Molecular Dynamics) were used.

Materials. 8-Azido-[α-^{32}P]ATP was purchased from Affinity Laboratories Inc. Acetone/ether-precipitated *E. coli* lipids were from Avanti Polar Lipids. Other chemicals were as in ref *33*.

RESULTS

General. The purpose of this work was to determine the role(s) of Gln-471 and Gln-1114 in the N- and C-terminal catalytic sites of Pgp, respectively. These glutamine residues are conserved in all ABC transporters, and three possible roles have been proposed (see the introduction), namely, (1) activation of the attacking water for ATP hydrolysis, (2) coordination of the essential cofactor Mg^{2+} in bound Mg nucleotide, and/or (3) signal communication between the chemical reaction in the catalytic site and coupling to drug transport. We constructed the mutants Q471A and -E and Q1114A and -E. The Ala mutants remove both NE2 and OE1 atoms, which are implicated in interaction with water molecules or Mg^{2+}, respectively, while the Glu mutants retain H-bonding capability. Making the equivalent mutations in both nucleotide sites enabled us also to look for possible "asymmetric" effects on function.

Mutagenesis and Protein Purification. Mutations were generated in mouse MDR3 protein and expressed in *P. pastoris* as described in Experimental Procedures. Mutant proteins were purified as in ref *33*. Briefly, the procedure consisted of cell disruption, preparation of crude microsomes, solubilization with DM, Ni-NTA chromatography, DEAE-cellulose chromatography, and pressure filtration concentration. Figure 1 shows an SDS gel of the final, purified, detergent-soluble, mutant proteins. The yield of the mutant protein was ∼0.03 mg/g wet wt of cells (wild type = 0.05); 3 L of flask culture yielded around 2 mg of pure protein. Before assays, the protein was activated by preincubation with lipid and DTT (*33*; see Experimental Procedures). Usually the lipid/protein ratio was 100/1, except where radioactivity measurements were carried out when the ratio was 2/1, to eliminate nonspecific binding. The latter ratio is sufficient for maximal activation of ATPase (*33*). At least two preparations of each mutant protein were made, and it was confirmed that the specific ATPase activity of different preparations agreed.

MgATPase Activities of Mutant Proteins. To determine whether the Gln residues are directly involved in reaction chemistry, ATP hydrolysis was assayed under different conditions. Table 1 shows specific MgATPase activities of wild-type and mutant proteins in the absence of drug (basal activity) and after stimulation by verapamil, tetraphenylphosphonium (TPP^+), and vinblastine. Each of these compounds is a well-established activator of Pgp ATPase activity (e.g.,

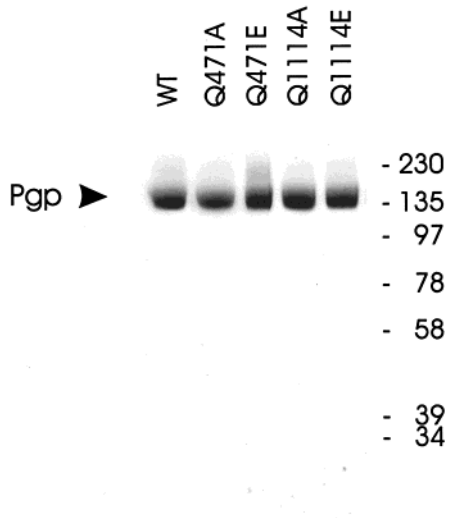

FIGURE 1: SDS gel of purified mutant and wild-type Pgp. Purified Pgp was run on a 10% SDS gel and stained with Coomassie Blue. 2 μg of protein was run in each lane.

refs *12*−*14*). The first column shows that basal activity was lower than wild type in all four mutant proteins. Verapamil stimulated activity by 13-fold in wild type, to a slightly lesser extent in the two Q → E mutants, and to a significantly lesser extent in the two Q → A mutants (column 2). The same pattern was seen with TPP^+, where stimulation in the Ala mutants was minimal (column 3). The results with vinblastine (column 4) were similar: wild type was stimulated by 8-fold, the two Glu mutants were somewhat less stimulated, and the two Ala mutants were stimulated only weakly. Therefore, impairment of drug stimulation of MgATPase was an obvious consequence of mutation. Column 5 of Table 1 shows that all four mutants had the same K_M(MgATP) as wild type.

The maximal specific MgATPase seen in the mutants with all three drugs was lower than in wild type. Table 2 presents these data as percentages of wild-type activity. The two Ala mutants had activities in the range of 1.3−4.3% of wild type, and the two Glu mutants had activities in the range of 6.2−9.7% of wild type. Consistently, the Ala mutants gave lower maximal activities than the Glu mutants.

We carried out a series of experiments in which we assayed MgATPase activity as a function of increasing verapamil or TPP^+ concentration, at both pH 7.5 and pH 8.5 (data not shown). There was essentially no difference between mutant proteins and wild type in the concentration of either drug required to give 50% stimulation of ATPase activity. Consonant with a recent report (*37*), lower concentrations of both drugs were required to elicit 50% stimulation of ATPase at pH 8.5 than at pH 7.5, and this was the case in both the mutant and the wild-type proteins. MgATPase activity in the presence of 150 μM verapamil was also assayed as a function of pH between 5.0 and 8.7. With wild-type the "pH profile" was similar to data published earlier (*13*, *14*), showing a broad pH optimum from 7.2 to 8.2 and a small decrease at pH 8.2−8.7 (data not shown). The pH at which half-maximal activity occurred in wild type was 6.05. The four mutants were each similar to wild type in pH profile (data not shown).

Mg^{2+} Dependence of ATPase Activity. To investigate whether the Gln residues are ligands to the Mg^{2+} in MgATP substrate, ATPase activity was assayed as a function of increasing $MgCl_2$ concentration, keeping NaATP constant at 10 mM in the presence of 150 μM verapamil. These data are shown in Figure 2A. Results for wild type were that maximal activity was achieved at an equimolar concentration ratio of Mg^{2+}/ATP, and a slight decrease in activity occurred at higher ratios. The concentration of Mg^{2+} at which half-maximal activity occurred was 1.27 mM. The four mutants showed the same relative dependence on Mg^{2+} concentration as in wild type (Figure 2B), with Mg^{2+} concentration for half-maximal activity as follows: Q471A, 1.95 mM; Q471E, 1.05 mM; Q1114A, 1.78 mM; Q1114E, 2.07 mM. These values were not significantly different from that of wild type.

Competitive Inhibition by MgADP. MgADP is known to be a competitive inhibitor of Pgp−ATPase activity with previously reported K_i values in hamster Pgp of 0.35 and 0.70 mM for plasma-membrane-located and purified reconstituted Pgp, respectively (*13*, *14*). Here we found that K_i-(MgADP) in wild-type purified mouse MDR3 Pgp was 0.15 mM. We assayed MgADP inhibition in the mutant proteins and found the following K_i(MgADP) values: Q471A, 0.055 mM; Q471E, 0.24 mM; Q1114A, 0.040 mM; Q1114E, 0.13 mM. Thus the two Glu mutants resembled each other and also the wild type, whereas the two Ala mutants resembled each other but were lower than wild type. If the natural Gln were a ligand to the Mg^{2+} in MgADP, one might have expected the Ala mutants to show higher K_i(MgADP) values than wild type, not lower.

Inhibition of MgATPase Activity by Vanadate, Fluoroaluminate, and Beryllium Fluoride. Figure 3 shows inhibition of wild-type and mutant proteins by the transition state analogue vanadate (Vi). IC_{50} (concentration yielding 50% inhibition) was 0.65 μM for wild type under the conditions used, and corresponding values in the mutants were as follows: Q471A, 6.7 μM; Q1114A, 10 μM; Q471E, 3.7 μM; Q1114E, 2.4 μM. Therefore, the IC_{50} was shifted to higher values in the mutants, with Ala mutants affected to greater extent than Glu mutants. Inhibition by the transition state analogue fluoroaluminate (AlFx) and the MgATP ground state analogue beryllium fluoride (BeFx) was also tested, at a single concentration of analogue that gave full inhibition in wild type (see Experimental Procedures), and it was seen that each of the mutant proteins was fully inhibited by AlFx and BeFx.

Vi Trapping of 8-Azido-[α-^{32}P]ADP. To investigate whether Gln-471 or Gln-1114 is required to form the catalytic transition state, Vi trapping experiments were performed. Wild-type and mutant proteins were preincubated with Vi and 8-azido-[α-^{32}P]ATP in the presence of EDTA or Mg^{2+} and then run through centrifuge columns to remove unbound ligands, and the eluate proteins were subjected to UV irradiation and SDS gel electrophoresis. Resultant autoradiograms are shown in Figure 4. It is seen that Vi trapping of 8-azido-[α-^{32}P]ADP was absent if divalent metal was removed by EDTA but that all of the four mutant proteins showed trapping with Mg^{2+}. This result establishes that the Gln-471 and Gln-1114 residues are not critical ligands to the Mg^{2+} in the transition state.

Photolabeling of Mutant Proteins by 8-Azido-[α-^{32}P]ATP. Mg-8-azido-ATP is a good hydrolysis substrate in Pgp (*10*). Wild-type and mutant Pgp were incubated with 8-azido-[α-^{32}P]ATP in the presence of 0 and then increasing

Table 1: MgATPase Activity of Mutant Proteins[a]

mutant Pgp	specific ATPase activity [μmol min^{-1} (mg of protein)$^{-1}$]			K_M(MgATP) (mM)
	no drug	plus verapamil[b]	plus TPP^{+} [b]	plus vinblastine[b]
wild type	0.32	4.0 (13×)	3.0 (9.4×)	2.6 (8.0×)	0.69
Q471A	0.033	0.10 (3.0×)	0.04 (1.2×)	0.04 (1.3×)	0.60
Q471E	0.029	0.33 (11×)	0.29 (10×)	0.22 (7.6×)	0.67
Q1114A	0.039	0.17 (4.4×)	0.08 (2.1×)	0.06 (1.6×)	0.70
Q1114E	0.029	0.26 (9.0×)	0.22 (7.6×)	0.16 (5.6×)	0.62

[a] ATPase activity was assayed as described in Experimental Procedures in the presence of 10 mM NaATP and 12 mM $MgCl_2$, with 150 μM verapamil or 300 μM TPP^{+} (added as bromide salt) or 30 μM vinblastine. These concentrations elicited maximal activity. K_M(MgATP) values were obtained in the presence of 150 μM verapamil. [b] Fold stimulation by drug in parentheses.

Table 2: Specific MgATPase Activity of Mutant Pgp Expressed as a Percentage of Wild-Type Activity

mutant Pgp	ATPase activity[a] (%)		
	plus verapamil	plus TPP^{+}	plus vinblastine
wild type	100	100	100
Q471A	2.5	1.3	1.5
Q471E	8.3	9.7	8.5
Q1114A	4.3	2.7	2.3
Q1114E	6.5	7.3	6.2

[a] The specific activity of wild type was set at 100, and the relative activity of each mutant is given (% of wild type). The concentration of drugs was as in Table 1 to achieve maximal activity.

concentrations of Mg^{2+}, subjected to UV irradiation, and run on SDS gels. Figure 5 shows the results obtained. It is apparent that the mutant proteins behaved similarly to the wild type. All showed low photolabeling in the absence of Mg^{2+}, which increased in the presence of increasing concentrations of Mg^{2+}. These data give further support to the view that the Ala and Glu mutations had no major effect on Mg nucleotide binding to the catalytic sites.

DISCUSSION

General. Gln-471 and Gln-1114 are equivalent residues in the two catalytic sites of P-glycoprotein, which are conserved in ABC transporters, implying an important function. X-ray structures show they are located in close proximity to the bound nucleotide in catalytic sites of HisP and RbsA. Three possible roles for these residues are likely (see the introduction), namely, (1) for activation of the attacking water for ATP hydrolysis, (2) as a coordinating ligand to the Mg^{2+} cofactor in Mg-nucleotide, and (3) in signal transduction between catalytic sites and drug transport sites. The work conducted here with the Pgp mutants Q471A and -E and Q1114A and -E was designed to examine these possibilities.

The Mutations Had No Apparent Effect on Substrate MgATP Binding or Drug Binding Parameters. For each of the mutants, K_M(MgATP) (Table 1) and photolabeling with Mg-8-azido-ATP (Figure 5) were the same as for wild type. Also, concentrations of verapamil or TPP^{+} required for half-maximal stimulation of ATPase activity were the same in wild type and in all four mutants (Results). Therefore, no major effects on either MgATP binding or drug binding parameters were apparent.

The Mutations Had No Major Effect on Reaction Chemistry. MgATPase activity was reduced in both Ala and Glu mutants, with the larger effects seen in the Ala mutants (Tables 1 and 2). In no case, however, was impairment

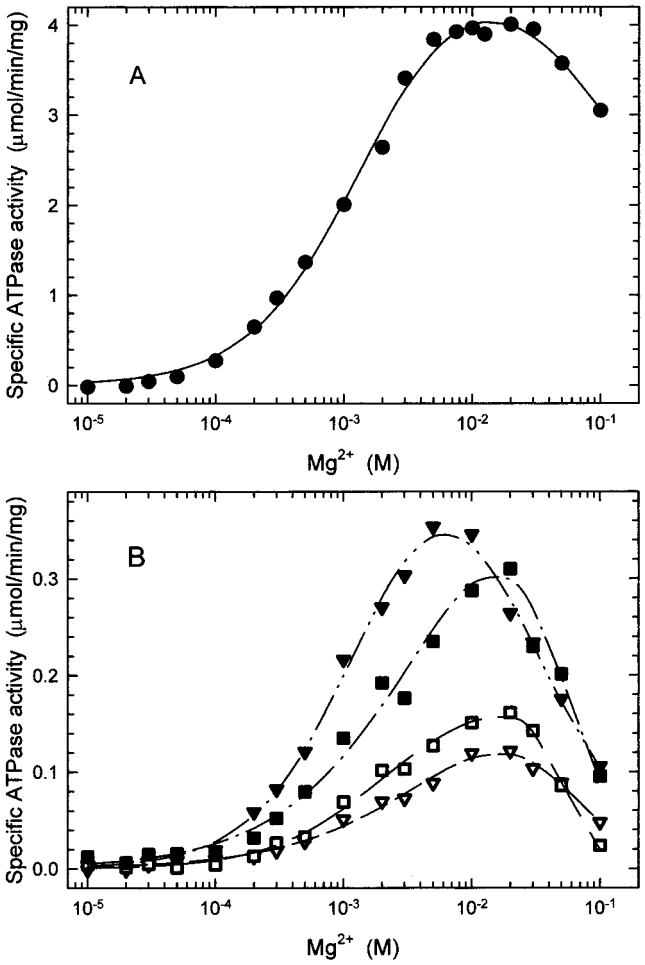

FIGURE 2: Dependence of ATPase activity of wild-type and mutant Pgp on Mg^{2+} concentration. ATPase was assayed in assay medium containing 10 mM NaATP and 150 μM verapamil, with increasing concentrations of $MgCl_2$. See Experimental Procedures for details. (A) Wild type (●). (B) Mutants: ▽, Q471A; ▼, Q471E; □, Q1114A; ■, Q1114E. Note the change in vertical scale in panel B versus panel A.

greater than by 2 orders of magnitude and in most cases less than this. No effects of the mutations on the pH dependence of the reaction were seen. Figure 4 showed that formation of the transition state of the reaction, as monitored by Vi trapping of Mg-8-azido-ADP, occurred in all of the mutants. These results are in complete contrast to earlier studies of, for example, conserved Walker A Lys or Ser residues or the Walker B Asp residue, where mutagenesis was found to completely abolish MgATPase and Vi trapping of MgADP or Mg-8-azido-ADP (*19*, *20*; Urbatsch et al., manuscript submitted). Therefore, the natural Gln residue is not required

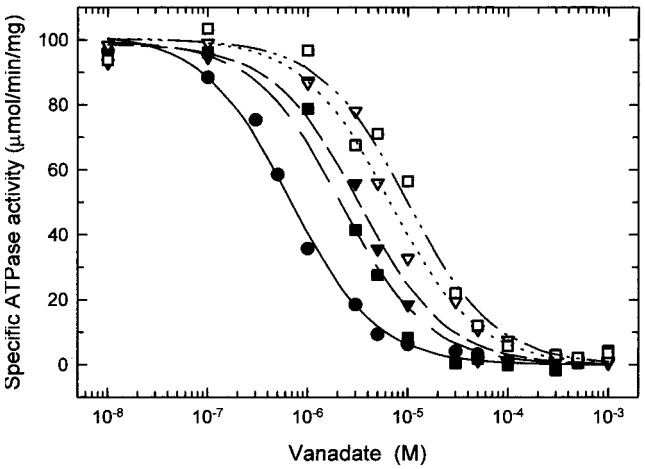

FIGURE 3: Inhibition of MgATPase activity of wild-type and mutant Pgp by Vi. Assay of MgATPase activity and its inhibition by Vi are described in Experimental Procedures. Key: ●, wild-type; ▽, Q471A; ▼, Q471E; □, Q1114A; ■, Q1114E.

for catalysis. Rather it seems likely that, in the mutants studied here, steps after formation of the transition state are slowed, resulting in lowered ATP hydrolysis rates.

In other ATPase enzymes, where a specific residue is required for orientation or activation of the attacking water, mutagenesis of that critical side chain has been seen to cause impairment by a factor of at least 10^3-fold (e.g., ref *38*). Therefore, we can state with confidence that neither Gln-471 nor Gln-1114 in Pgp is the sole residue responsible for positioning or activation of the attacking water. One could suggest that the Gln residue acts in concert with another residue to position the attacking water (such a suggestion was made in ref *31* in regard to the homologous Gln-100 of HisP). While we cannot dismiss this possibility, the very high level of sequence conservation of this residue in ABC transporters and the retention of significant hydrolysis activity in the mutants reported here suggest that another function is present.

Mutation of the Gln Residues Had No Measurable Effects on Interaction with the Mg^{2+} Cofactor. K_M(MgATP) (Table 1) and the dependence of ATPase activity on Mg^{2+} concentration (Figure 2) were the same in the Ala and Glu mutants as in wild type. Vi trapping of the transition state occurred in mutants and wild type with Mg-8-azido-ADP (Figure 4). In sharp contrast, when another Pgp residue (Ser-430 and Ser-1073) that provides a putative Mg^{2+} coordination ligand in catalytic sites was recently mutated to Ala (*41*), MgATPase activity and Vi trapping of MgADP or Mg-8-azido-ADP were abolished. This leads us to conclude that Gln-471 and Gln-1114 do not interact with Mg^{2+} of the Mg

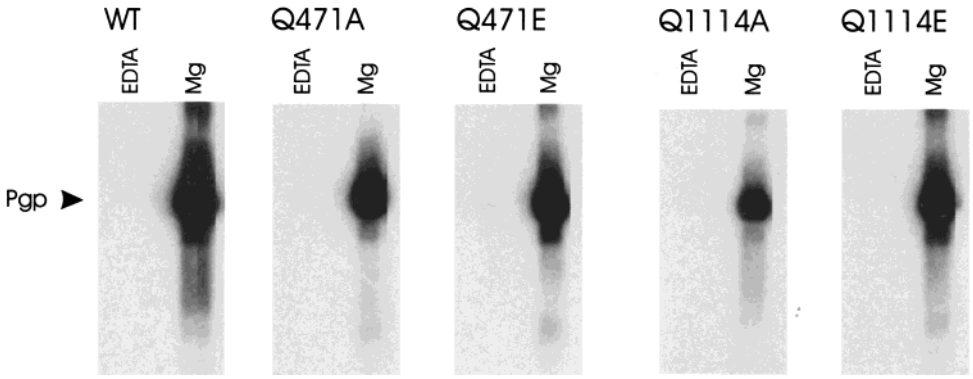

FIGURE 4: Vi trapping of 8-azido-[α-^{32}P]ADP in mutant and wild-type Pgp. Mutant and wild-type Pgp were preincubated with Vi and 8-azido-[α-^{32}P]ATP in the presence of EDTA (1 mM) or $MgCl_2$ (3 mM), then unbound ligands were removed by passage through centrifuge columns, and the eluates were subjected to UV irradiation, SDS gel electrophoresis, and autoradiography. See Experimental Procedures for details.

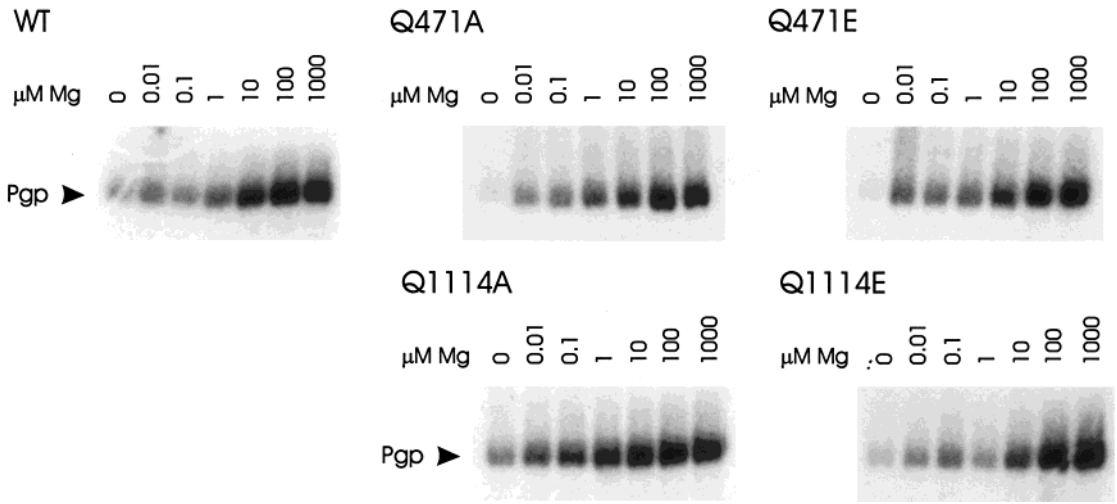

FIGURE 5: Direct photolabeling of wild-type and mutant Pgp by 8-azido-[α-^{32}P]ATP. Wild-type and mutant Pgp were incubated with 8-azido-[α-^{32}P]ATP in the presence of 0 (contained 1 mM EDTA) or increasing concentrations of $MgCl_2$ as shown. Samples were subjected to UV irradiation, SDS gel electrophoresis, and autoradiography. For details, see Experimental Procedures.

nucleotide at the stages of MgATP binding or at the step of the transition state. Assays of K_i values for competitive inhibition by MgADP in mutants as compared to wild type (Results) showed that, at least in Pgp, Gln-471 and Gln-1114 are not important Mg^{2+} ligands for the product MgADP.

The Most Obvious Effect of Mutation Was To Reduce Stimulation of ATPase Activity by Drugs. Stimulation of MgATPase activity by the drugs verapamil, TPP^+, and vinblastine was strongly and consistently impaired in the Ala mutants and impaired to a lesser effect, but still consistently, by the Glu mutations (Table 1). The Glu mutation would tend to conserve H-bonding features of the Gln better than the Ala mutation; thus the better retention of function in the Glu mutants could well be related to this factor. A reasonable conclusion is that Gln-471 and Gln-1114 are involved in interdomain communication, transmitting conformational signals out of the catalytic site as the ATP hydrolysis transition state forms and collapses, to the coupling mechanism that ultimately generates transport-related conformational changes at the drug-binding sites. ATPase activity was reduced in the mutants to a few percent of wild type (Ala > Glu, Table 2). This can be explained as due to interruption, by the mutations, of the signal transmission path, which is likely tightly coupled to catalytic turnover. Inhibition of ATPase activity by Vi was somewhat affected in the mutants, as judged by increased IC_{50} for Vi (Ala > Glu, Figure 3). This could also be due to partial disruption by the mutations of the transmission system to which the transition state is normally "geared".

Equivalent Mutations in Either of the Two Pgp Catalytic Sites Gave "Symmetric" Functional Consequences. Over the range of different activities assayed here, we found consistently that the two equivalent Ala mutations gave broadly the same functional consequences, and this was true also of the two Glu mutants. Thus, the two nucleotide-binding domains behaved "symmetrically". We have observed this previously in a mutagenesis study of the Walker A Ser residues (*41*). Others noted similar findings, although based on less extensive series of assays (*19*, *20*). However, asymmetric behavior of the two nucleotide sites has also been reported (*39*, *40*). This point is important in relation to distinguishing possible different contributions of the two catalytic sites to the overall mechanism (*30*). Our data indicate that the two nucleotide-binding domains do not differ in their functions.

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