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)

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ABSTRACT: The human *MDR*1 (ABCB1) gene product, P-glycoprotein (Pgp), functions as an ATP-dependent efflux pump for a variety of chemotherapeutic drugs. In this study, we assessed the role of conserved glutamate residues in the Walker B domain of the two ATP sites (E556 and E1201, respectively) during the catalytic cycle of human Pgp. The mutant Pgps (E556Q, E556A, E1201Q, E1201A, E556/1201Q, and E556/1201A) were characterized using a vaccinia virus based expression system. Although steady-state ATP hydrolysis and drug transport activities were abrogated in both E556Q and E1201Q mutant Pgps, [α-^{32}P]-8-azidoADP was trapped in the presence of vanadate (Vi), and the release of trapped [α-^{32}P]-8-azidoADP occurred to a similar extent as in wild-type Pgp. This indicates that these mutations do not affect either the first hydrolysis event or the ADP release step. Similar results were also obtained when Glu residues were replaced with Ala (E556A and E1201A). Following the first hydrolysis event and release of [α-^{32}P]-8-azidoADP, both E556Q and E1201Q mutant Pgps failed to undergo another cycle of Vi-induced [α-^{32}P]-8-azidoADP trapping. Interestingly, the double mutants E556/1201Q and E556/1201A trapped [α-^{32}P]-8-azidoADP even in the absence of Vi, and the occluded nucleotide was not released after incubation at 37 °C for an extended period. In addition, the properties of transition state conformation of the double mutants generated in the absence of Vi were found to be similar to that of the wild-type protein trapped in the presence of Vi (Pgp·[α-^{32}P]-8-azidoADP·Vi). Thus, in contrast to the single mutants, the double mutants appear to be defective in the ADP release step. In aggregate, these data suggest that E556 and E1201 residues in the Walker B domains may not be critical as catalytic carboxylates for the cleavage of the bond between the γ-P and the β-P of ATP during hydrolysis but are essential for the second ATP hydrolysis step and completion of the catalytic cycle.

The ATP-binding cassette (ABC)[1] family of transport proteins is one of the largest families of proteins in living organisms (*1*, *2*). The functional unit of these transporters is comprised of two nucleotide-binding domains (NBDs) and two transmembrane domains, containing six putative α-helices (*3*, *4*). The NBD or ATP site consists of the highly conserved Walker A and B motifs and the ABC signature or the linker region (*5*, *6*). ABC transporters utilize energy for transporting substrates across membranes, and an understanding of how ATP hydrolysis is coupled to transport is vital to elucidating their mechanism (*7*). Due to the close sequence homology between the ABC transporters, particularly that within the NBDs, it is possible that these transporters may exhibit analogous mechanisms (*8*, *9*). P-glycoprotein (Pgp), an archetypical ABC transporter and one of the most extensively studied (*4*−*6*), would be an excellent candidate to discern such universal mechanisms common to this family of proteins.

A recent study analyzed the functional consequences of mutating 14 of the most conserved acidic residues in mouse Mdr3 (also known as *mdr*1a), one of two mouse homologues to human *MDR*1 (*10*). Of the 14 residues studied those whose mutation resulted in the most dramatic phenotype were the pairs (in the NBD1 and NBD2, respectively) D551/1196 and E552/1197 in the conserved Walker B (I, L, L, L, D, E) domain. The Walker A and B and the signature (linker) region residues are identical in human MDR1 and mouse, rat, and hamster Mdr1 and Mdr3 (see supplement to ref *10*). Previous work has demonstrated that the pair D551/1196 (equivalent to D555 and D1200 in human Pgp) is implicated in the coordination of Mg^{2+} in both mouse and human Pgp (*11*, *12*). There is, however, no clear consensus about the role of the conserved glutamate residues E552 and E1197 (equivalent to E556 and E1201 in human Pgp). The analyses of the structure of NBDs of some of the ABC and analogous transport proteins (*13*−*20*) have suggested that these conserved glutamates are the most likely candidates to play the role of the catalytic carboxylate. Thus it is speculated that

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[1] Abbreviations: ABC, ATP-binding cassette; BeF_x, beryllium fluoride; IAAP, [^{125}I]iodoarylazidoprazosin; NBD, nucleotide-binding domain; PAGE, polyacrylamide gel electrophoresis; PP_i, sodium pyrophosphate; Pgp, P-glycoprotein; Vi, orthovanadate.

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these residues activate a water molecule, which will attack the $\beta-\gamma$-phosphate bond of ATP. Urbatsch et al. found that the mutants E552Q and E1197Q were able to trap [α-^{32}P]-8-azidoADP in the presence of Vi but showed no drug-stimulated steady-state ATPase activity (*10*). Results with mutations in either the N- or the C-ATP site alone are difficult to interpret because the second ATP site is fully functional. Second, in investigating the role of the conserved glutamates as an activating base, the mutation of E → Q alone would not provide sufficient evidence to arrive at an unequivocal conclusion as the glutamine can also fulfill a role similar to the glutamate (*13*, *21*). In this study, in addition to the mutants E556Q and E1201Q we have also characterized the E556A and E1201A as well as the double (E556/1201Q and E556/1201A) mutant Pgps.

We demonstrate in this paper that in human Pgp residues E556 and E1201 do not appear to have a role in the cleavage of the bond between the γ-P and β-P of ATP per se and do not function as catalytic carboxylates since E556 → Q/A and E1201 → Q/A mutants exhibit Vi-induced occlusion of 8-azidoADP following hydrolysis of 8-azidoATP. However, as mutations in these residues are detrimental to function, they must perform a critical task(s). We therefore explored in detail the role of these residues. We have previously reported that there are two ATP hydrolysis events in a single catalytic cycle, one involved in drug transport and the other in resetting the Pgp molecule following the conformational change that accompanies the transport step (*22*, *23*). The results of this study show that the E556Q and E1201Q mutant Pgps hydrolyze ATP and allow normal release of ADP during the first step but are defective in the second ATP hydrolysis event, and as a result of which, both steady-state ATP hydrolysis and drug transport activities are abrogated [part of this work has appeared in abstract form (*24*)]. We suggest that E556 and E1201 residues may reside in a "switch" region of Pgp comprised of the D loop and the ABC signature region, based on the model of MalK and Btu D NBD dimers (*25*, *26*), which ensures that following the first ATP hydrolysis reaction at either the N- or the C-NBD, the alternate site is recruited for the next hydrolysis event. These data also provide strong evidence for the requirement of a second ATP hydrolysis step for the completion of a single catalytic cycle of Pgp. Finally, we observed that the Pgp double mutant (E556/1201Q or E556/1201A) traps [α-^{32}P]-8-azidoADP even in the absence of Vi. We assessed the properties of this transition state extensively and determined that it was comparable to the Pgp·ADP·Vi transition state of wild-type Pgp. This validates the central premises of the use of Vi to study the catalytic cycle of Pgp: that the Pgp·ADP·P_i and the Pgp·ADP·Vi complexes are equivalent and that the transition state, Pgp·ADP·P_i, represents an intermediate state during the normal reaction pathway (*27*).

EXPERIMENTAL PROCEDURES

Chemicals. Calcein-AM, bodipy-FL-vinblastine, and rhodamine 123 were purchased from Molecular Probes, Inc. (Eugene, OR). Cyclosporin A was purchased from Calbiochem (San Diego, CA). [^{125}I]Iodoarylazidoprazosin (IAAP), 2200 Ci/mmol, was obtained from PerkinElmer Life Sciences (Boston, MA). [α-^{32}P]-8-AzidoATP (15−20 Ci/mmol), [α-^{32}P]-8-azidoADP (15−20 Ci/mmol), [γ-^{32}P]-8-azidoATP (15−20 Ci/mmol), 8-azidoATP, and 8-azidoADP were purchased from Affinity Labeling Technologies, Inc. (Lexington, KY). The [α-^{32}P]-8-azidoATP showed no detectable contaminating [α-^{32}P]-8-azidoADP using thin-layer chromatography with 0.8 M LiCl as the solvent. Pgp-specific monoclonal antibody C219 was obtained from Fujirebio Diagnostics Inc. (Malvern, PA). Human Pgp-specific monoclonal antibody MRK 16 was purchased from Kyowa Medex Co., Ltd. (Tokyo, Japan). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Vaccinia Virus Expression Vector Constructs. Specific point mutations were constructed by sequence overlap PCR using pTM1-*MDR*1 (wild type) DNA as the expression vector template as described previously (*12*, *28*). The coding sequence for the E556Q mutant primer was 5′-ATC CTC CTG CTG GAT CAG GCC ACG TCA GCC TTG-3′; for the E1201Q mutant primer, 5′-ATT TTG CTT TTG GAT CAA GCC ACG TCA GCT CTG-3′; for the E556A mutant primer, 5′-ATC CTC CTG CTG GAT GCG GCC ACG TCA GCC TTG-3′; and for the E1201A primer, 5′-ATT TTG CTT TTG GAT GCA GCC ACG TCA GCT CTG-3′. The DNA sequence of all constructs was verified in both directions by automated sequencing with the PRISM Big Dye Terminator Sequencing Kit (PerkinElmer Corp., Norwalk, CT).

Cell-Surface Expression of Wild-Type and Mutant Pgps and Drug Accumulation Assays by Flow Cytometry. HeLa cells infected with vTF7-3 and transfected with vector pTM1-*MDR*1 wild type or the vector pTM1-*MDR*1 mutants were stained with the external epitope-specific monoclonal antibody, MRK 16 (*29*), and analyzed on a FACSort flow cytometer using CellQuest software (Becton Dickinson FACS system, San Jose, CA) as described (*30*, *31*). Fluorescent drug accumulation assays were performed by flow cytometry in intact infected−transfected HeLa cells. The calcein accumulation assay was performed by adding calcein-AM, 0.5 μM, to 250000 cells in a reaction mixture of 4.5 mL and incubated at 37 °C for 10 min, centrifuged for 5 min at 500*g*, and transferred to ice. The pellet was resuspended in PBS + 0.1% BSA and analyzed by FACSort as described (*28*, *30*, *31*). The rhodamine 123 efflux assay was initiated by first incubating 250000 intact infected−transfected HeLa cells with 0.5 μM rhodamine 123 for 45 min at 37 °C to accumulate the drug into the cells. Excess drug was washed off by centrifugation at 500*g* for 5 min, and the cells were resuspended in IMDM + 5% FBS and incubated at 37 °C for 30 min to allow the cells to efflux the accumulated rhodamine 123. Following centrifugation for 5 min at 500*g* the pellet was resuspended in PBS + 0.1% BSA and analyzed by FACSort.

Preparation of Crude Membranes from HeLa Cells Infected−Transfected with pTM1 Vector Carrying the Wild-Type and Mutant Human MDR1 Genes. Crude membranes were prepared from vTF7-3 infected HeLa cells transfected with vector pTM1-*MDR*1 wild type, pTM1-*MDR*1-E556Q, pTM1-*MDR*1-E1201Q, pTM1-*MDR*1-E556/1201Q, pTM1-*MDR*1-E556A, pTM1-*MDR*1-E1201A, and pTM1-*MDR*1-E556/1201A as described previously (*28*, *31*) and stored at −70 °C. Total protein was quantified by the Amido Black protein estimation method as previously described (*32*), and Pgp expression level was determined by immunoblot analysis using the monoclonal antibody C219 (*30*, *33*).

Photoaffinity Labeling with IAAP. The crude membranes (50−70 μg of protein) were incubated with IAAP (10 nM)

for 5 min under subdued light and exposed to UV light (365 nm) as described (*22*) for 10 min at 21−23 °C. Samples were then immunoprecipitated as described below. In some experiments (as indicated in the figure legend), intact HeLa cells expressing wild-type and mutant Pgps were also labeled with IAAP. The 500000 cells in IMDM medium containing 5% FBS were incubated with IAAP (10 nM) for 5 min under subdued light and exposed to UV light (365 nm) for 10 min at 21−23 °C. The cells were centrifuged at 500*g* for 5 min, and the supernatant was discarded. Cells were then resuspended in 100 μL of TD buffer [10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100 (v/v), 10 mM $MgSO_4$, 2 mM $CaCl_2$, 1% (v/v) aprotinin, 2 mM AEBSF, 1 mM DTT, and 20 μg/mL micrococcal nuclease], incubated at 37 °C for 5 min, and quick-frozen in dry ice. The samples were then thawed and sonicated three times in a bath sonicator (1 min per sonication). Samples were then transferred to ice and immunoprecipitated as described below. Following SDS−PAGE the gels were dried and exposed to X-ray film, and in addition, the radioactivity incorporated into the Pgp band was estimated using the STORM 860 phosphorimager system (Molecular Dynamics, Sunnyvale, CA) using the software ImageQuaNT. In phosphorimage analysis radioactivity was obtained as arbitrary units.

Vanadate-Induced 8-AzidoADP Trapping in Pgp. The Vi-induced trapping was carried out as described previously (*22*, *23*) with minor modifications in some experiments. Crude membranes were incubated in the ATPase assay buffer (40 mM MES−Tris-HCl, pH 6.8, 50 mM KCl, 1 mM ouabain, 5 mM sodium azide, 1 mM EGTA, 2 mM DTT, and 5 mM $MgCl_2$) with the indicated nucleotides and 0.25 mM Vi in the dark at 37 °C for 10 min (in the experiment depicted in Figure 2 the reaction was allowed to proceed for varying times). One of the following nucleotides (as indicated in the figure legends) was used in the different experiments: 50 μM [α-^{32}P]-8-azidoATP, 50 μM [α-^{32}P]-8-azidoADP, or 50 μM [γ-^{32}P]-8-azidoATP (2 μCi/nmol). To demonstrate drug-stimulated Vi-induced trapping, 50 μM verapamil was added to the reaction mixture prior to addition of [α-^{32}P]-8-azidoATP, and 5 μM [α-^{32}P]-8-azidoATP was used in lieu of the 50 μM used in other experiments, as stated in the figure legends (see Results section and ref *10* for the rationale). All Vi-induced trapping reactions were stopped by the addition of 10 mM ice-cold ATP and placing the sample on ice. For experiments using [γ-^{32}P]-8-azidoATP, each sample was accompanied by a control, which was not photo-cross-linked to correct for incorporation of the ^{32}P-labeled γ-phosphate into Pgp by kinase-mediated phosphorylation. To determine the distribution of [α-^{32}P]-8-azidoADP in the N- and the C-ATP site of Pgp, the samples were subjected to mild trypsin digestion following photo-cross-linking as described previously (*12*).

Binding of [α-^{32}P]-8-AzidoATP or [γ-^{32}P]-8-AzidoATP to Pgp. Crude membranes (50−70 μg of protein) were incubated with 10 μM [α-^{32}P]- or [γ-^{32}P]-8-azidoATP (10 μCi/nmol) for 5 min on ice in the ATPase assay buffer, photo-cross-linked, and then immunoprecipitated as described below.

ATPase Assay. The Vi-sensitive ATPase activity of wild-type and mutant Pgps was determined by using the P_i release assay as described previously (*23*, *34*, *35*).

Immunoprecipitation of Pgp. Following photo-cross-linking (50−70 μg of crude membrane protein in a reaction volume of 100 μL) 800 μL of ice-cold RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 1% aprotinin) was added to the reaction mixture. Pgp was immunoprecipitated by incubating with 12 μL of Pgp-specific monoclonal antibody C219 (1 mg/mL) for 3 h at 4 °C. Subsequent steps were carried out as described previously for immunoprecipitation using the polyclonal antibody PEPG 13 (*28*).

RESULTS

Substitution of the Highly Conserved Residues E556 and E1201 with Q in the Walker B Domain of the ATP Sites of Pgp Does Not Affect Cell Surface Expression but Abrogates Transport Function. In this study we aim to understand the role of the highly conserved glutamates within the Walker B region of the N- and the C-ATP sites in the mechanism of ATP hydrolysis by Pgp. We generated the single mutants E556Q and E1201Q and the double mutant E556/1201Q of human Pgp and characterized them in a Vaccinia virus based expression system. HeLa cells infected−transfected with the wild-type and three mutant Pgps showed comparable cell surface expression measured by staining with human Pgp-specific monoclonal antibody MRK 16 in a FACS assay (Figure 1A). The functional status of the wild-type and mutant Pgps was assessed by using the calcein-AM efflux assay. Figure 1B illustrates that in a flow cytometry assay HeLa cells expressing wild-type Pgp show reduced accumulation of fluorescent calcein, as compared to cells infected−transfected with the pTM1 vector alone. However, cells expressing equivalent amounts of E556Q, E1201Q, and E556/1201Q mutant Pgps accumulate high levels of calcein comparable to the HeLa cells infected with the control pTM1 vector. Similar results were also obtained with rhodamine 123 and bodipy-FL-vinblastine (data not given), indicating that the mutant Pgps are defective in the transport of a number of fluorescent substrates. Additionally, crude membranes prepared from HeLa cells expressing the wild-type Pgp showed a basal ATPase activity of 9.4 nmol of P_i min^{-1} (mg of protein)$^{-1}$, which was enhanced ∼2.5-fold to 23 nmol of P_i min^{-1} (mg of protein)$^{-1}$ in the presence of 50 μM verapamil. These values are consistent with earlier published reports (*30*, *31*). However, all three mutant Pgps did not show Vi-sensitive basal or verapamil-stimulated ATPase activity, though immunoblotting with the Pgp-specific monoclonal antibody C219 showed similar levels of Pgp in crude membranes of HeLa cells expressing the wild-type and mutant proteins (see inset to Figure 1A).

Wild-Type, E556Q, E1201Q, and E556/1201Q Pgps Exhibit Differences in the Trapping of [α-^{32}P]-8-AzidoADP. The wild-type and all three mutant Pgps bind [α-^{32}P]-8-azidoATP, and this binding is specific as it is competed by the addition of a 100-fold excess of nonradioactive ATP (data not given). The nucleotide analogue 8-azidoATP is a good hydrolysis substrate for Pgp and follows Michaelis−Menten kinetics with a K_m of 400−600 μM, which is comparable to the K_m for ATP (330 μM) under identical conditions (*36*). The maximal velocity of the hydrolysis reaction is about 8−10-fold slower with 8-azidoATP compared to ATP (*36*). Furthermore, [α-^{32}P]-8-azidoATP is a useful photoaffinity

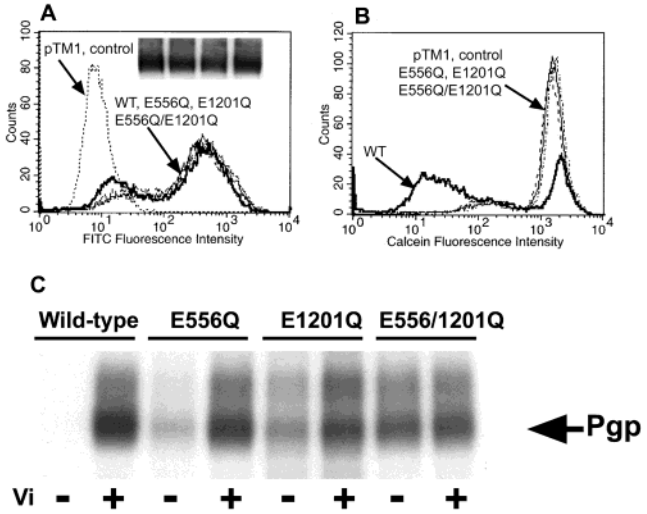

FIGURE 1: Cell surface expression, substrate transport, and Vi-induced trapping in wild-type and mutant Pgps, E556Q, E1201Q, and E556/1201Q. (A) Replacement of the highly conserved residues E556 and E1201 with Q in the nucleotide-binding domain of Pgp does not affect cell surface expression. The cell surface expression of wild-type and mutant Pgps was determined by staining infected−transfected HeLa cells with human Pgp-specific monoclonal antibody, MRK 16, as described (*30*, *31*). The inset shows an immunoblot of crude membranes (10 μg of protein) prepared from infected−transfected HeLa cells using the Pgp-specific monoclonal antibody C219. The lanes represent (from left to right) wild-type Pgp and the mutant Pgps E556Q, E1201Q, and E556/1201Q, respectively. (B) Replacement of the highly conserved residues E556 and E1201 with Q in the nucleotide-binding domain of Pgp abolishes substrate transport. The transport assay was performed in infected−transfected HeLa cells using the Pgp substrate calcein-AM (0.5 μM) and analyzed by flow cytometry as described in the Experimental Procedures. Panels A and B show HeLa cells infected with vTF7-3 and transfected with vector pTM1 (control) or pTM1-*MDR*1 wild type (WT), pTM1-*MDR*1-E556Q (E556Q), pTM1-*MDR*1-E1201Q (E1201Q), and pTM1-*MDR*1-E556/1201Q (E556Q/E1201Q, double mutant). (C) Vi-induced trapping of [α-^{32}P]-8-azidoADP into wild-type and mutant Pgps. Crude membranes (60 μg of protein) were incubated with 50 μM [α-^{32}P]-8-azidoATP (2 μCi/nmol) in the absence (−) or presence (+) of 0.25 mM Vi for 10 min at 37 °C in the ATPase assay buffer. The reaction was stopped by adding 10 mM ice-cold ATP and transferring to ice. Samples were photo-cross-linked, immunoprecipitated with the Pgp-specific monoclonal antibody C219, and electrophoresed on an 8% gel. The dried gel was exposed to an X-ray film for 8−18 h at −70 °C. The lanes are labeled on the autoradiogram.

reagent that has helped to circumvent the technical difficulties associated with the low affinity of Pgp for the nucleotide (*37*, *38*). The experimental approaches chiefly rely on the Vi-induced trapping of [α-^{32}P]-8-azidoADP into Pgp followed by photo-cross-linking. This reaction also exhibits Michaelis−Menten kinetics with $K_m \simeq 20$ μM (*36*, *39*), and we have demonstrated that results obtained with photo-cross-linking experiments are comparable to those obtained by direct quantification of occluded [α-^{32}P]-8-azidoADP by using a liquid scintillation counter in the absence of photo-cross-linking (see Figure S2 in the supplement to ref *40*). We studied Vi-induced trapping of [α-^{32}P]-8-azidoADP in the wild-type and mutant Pgps. Consistent with previous findings, we demonstrate in Figure 1C that when wild-type Pgp is incubated with [α-^{32}P]-8-azidoATP alone at 37 °C, the radiolabel can be competed out by the excess nonradioactive ATP added at the end of the assay but prior to exposure to UV light. However, the transition state intermediate, Pgp·[α-^{32}P]-8-azidoADP·Vi generated in the presence of Vi cannot be competed out even with a 200-fold excess of ATP. Both of the mutant Pgps, E556Q and E1201Q, exhibit enhanced trapping of [α-^{32}P]-8-azidoADP in the presence of Vi. The mutant E1201Q Pgp shows a higher level of trapping compared to the E556Q protein in the absence of Vi. However, interestingly, the double mutant E556/1201Q shows the same level of trapping of [α-^{32}P]-8-azidoADP in the absence or presence of Vi (lanes 7 and 8 from the left in Figure 1C). Phosphorimager analysis of the gel depicted as an autoradiogram in Figure 1C shows that the mutant Pgps E556Q, E1201Q, and E556/1201Q trapped 26%, 43%, and 96% 8-azidoADP in the absence of Vi compared to that observed in the presence of Vi. In contrast, the wild-type Pgp shows <1% of the signal in the absence of Vi. The results with the single mutants E556Q and E1201Q are consistent with those obtained with the equivalent mutants (E552Q and E1197Q) in mouse Mdr3 (*10*). The double mutant (E552/1197Q) in mouse Mdr3 was not studied previously.

The E556Q and E1201Q Mutant Pgps Are Defective in Repeated Cycles of Vi-Induced [α-^{32}P]-8-AzidoADP Trapping and Release. Although both the wild-type and mutant (E556Q and E1201Q) Pgps exhibit Vi-induced [α-^{32}P]-8-azidoADP trapping, the mutant Pgps show a complete loss of steady-state ATP hydrolysis and transport function. A similar result was obtained previously with E552Q and E1197Q in mouse Mdr3 (*10*), and the authors speculated that these mutants hydrolyze ATP but cannot release either one of the products (ADP or P_i). Moreover, a subsequent study has extended this work and developed a model for the ATP hydrolysis cycle of Pgp based on this assumption (*41*). To identify the defective step in these mutants, we studied the time course of Vi-induced trapping and release of 8-azidoADP. The experiment in Figure 2A (panel I) demonstrates that within 10 min wild-type Pgp and the mutants E556Q and E1201Q show a saturating and similar level of Vi-induced trapping of [α-^{32}P]-8-azidoADP. To test the hypothesis that these mutants are defective in the ADP or P_i release step, we incubated wild-type and mutant Pgps with [α-^{32}P]-8-azidoATP in the presence of Vi for 10 min at 37 °C to allow trapping of [α-^{32}P]-8-azidoADP, washed off excess [α-^{32}P]-8-azidoATP and Vi, and monitored the fate of the trapped [α-^{32}P]-8-azidoADP over time. The results of this experiment depicted in Figure 2A (panel II) demonstrate that in the mutant Pgp, E556Q, the release [α-^{32}P]-8-azidoADP occurs at the same rate as the wild-type Pgp. In the mutant E1201Q Pgp, the release of nucleoside diphosphate is slightly slower, but the extent of release is comparable to wild-type Pgp. Thus, it is not the failure to release the nucleoside diphosphate that renders these mutants nonfunctional. Additionally, as Vi-induced trapping of ADP can occur only after release of P_i, it is unlikely that this step is defective in these mutants. We therefore continued to follow the subsequent steps in the catalytic cycle of Pgp (see ref *23* for a detailed description of this experimental strategy). Following release of trapped [α-^{32}P]-8-azidoADP, we added fresh [α-^{32}P]-8-azidoATP and Vi to initiate the next ATP hydrolysis event and followed the Vi induced trapping over time. We observed (Figure 2A, panel III) that while the wild-type Pgp exhibits a second Vi-induced trapping of [α-^{32}P]-8-azidoADP comparable to the first Vi-induced trapping

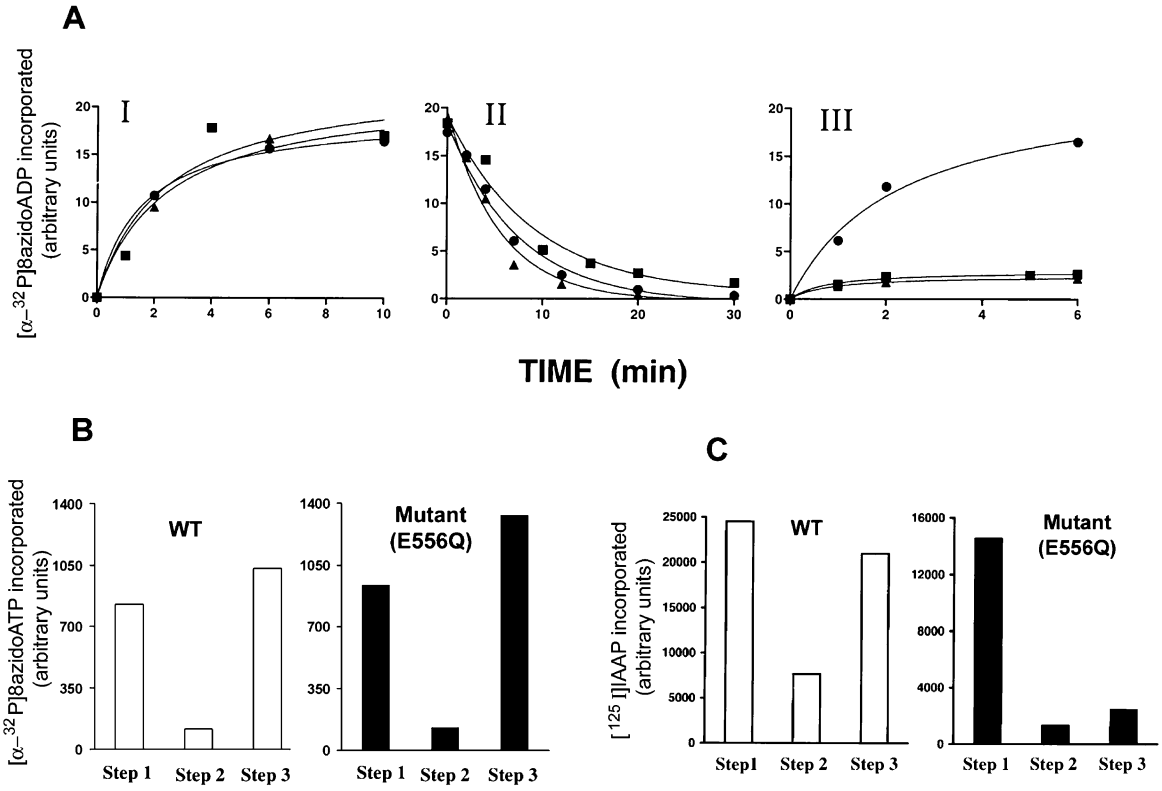

FIGURE 2: Analyses of the various steps in the catalytic cycle of wild-type and E556Q and E1201Q mutant Pgps. (A) Time course of Vi-induced trapping, release, and retrapping of [α-^{32}P]-8-azidoADP into wild-type, E556Q, and E1201Q Pgps. Panel I: Crude membranes were incubated with 50 μM [α-^{32}P]-8-azidoATP (2 μCi/nmol) and 0.25 mM Vi at 37 °C in the ATPase assay buffer. Aliquots (60 μg of protein) were removed at the indicated time points, and the samples were photo-cross-linked, immunoprecipitated, and electrophoresed as described in the Experimental Procedures. The dried gel was then exposed to a phosphorimager screen, and the intensity of the ^{32}P signal in the Pgp band was quantified as described (*22*). The graph shows the time course of [α-^{32}P]-8-azidoADP incorporation in the presence of Vi. Panel II: Crude membranes were incubated with 50 μM [α-^{32}P]-8-azidoATP (2 μCi/nmol) and 0.25 mM Vi in the ATPase assay buffer for 10 min at 37 °C. The reaction was stopped by adding 10 mM ice-cold ATP and transferring to ice, and the samples were centrifuged at 300000*g* for 15 min at 4 °C. The pellet was resuspended in the ATPase assay buffer and incubated at 37 °C. Aliquots (60 μg of protein) were removed at the indicated times and processed as in panel I. The graph shows the time course of [α-^{32}P]-8-azidoADP release. Panel III: Crude membranes were incubated with 50 μM [α-^{32}P]-8-azidoATP (2 μCi/nmol) and 0.25 mM Vi for 10 min at 37 °C, excess [α-^{32}P]-8-azidoATP and Vi were washed by centrifugation, and the trapped [α-^{32}P]-8-azidoADP was released by incubating at 37 °C as described above. A second cycle of Vi-induced trapping was then initiated at 37 °C by adding 50 μM [α-^{32}P]-8-azidoATP (2 μCi/nmol) and 0.25 mM Vi. Aliquots (60 μg of protein) were removed at the indicated times and processed as in panel I. The graph shows the time course of the second Vi-induced [α-^{32}P]-8-azidoADP trapping. Key for panels I, II, and III: (●) Pgp wild-type, (▲) Pgp-E556Q, and (■) Pgp-E1201Q. (B) Binding of [α-^{32}P]-8-azidoATP to wild-type and E556Q Pgp at various steps in the catalytic cycle. Crude membranes were incubated with 1 mM 8-azidoATP and 0.25 mM Vi at 37 °C in the ATPase assay buffer for 10 min at 37 °C. The reaction was stopped by adding 10 mM ice-cold ATP and transferring to ice, and the samples were centrifuged at 300000*g* for 15 min at 4 °C. The pellet was resuspended in the ATPase assay buffer and incubated at 37 °C for an additional 15 min. Three aliquots were removed (i) before the addition of 8-azidoATP, (ii) immediately following centrifugation, and (iii) at the end of the second incubation at 37 °C. [α-^{32}P]-8-AzidoATP (10 μM, 10 μCi/nmol) was added to each of these aliquots, and the mixture was incubated at 4 °C for 5 min and then photo-cross-linked, immunoprecipitated, and electrophoresed as described in the Experimental Procedures. The incorporation of labeled nucleotide was quantified using a phosphorimager as described previously (*22*). The indicated steps represent the following: step 1, [α-^{32}P]-8-azidoATP binding in the absence of any treatment; step 2, [α-^{32}P]-8-azidoATP binding following Vi-induced trapping of 8-azidoADP; and step 3, [α-^{32}P]-8-azidoATP binding after allowing release of 8-azidoADP trapped during the first ATP hydrolysis (*23*). (C) Binding of IAAP to wild-type and E556Q Pgp during different steps in the catalytic cycle. Crude membranes were treated as described in (B), except that at step 3 (release of 8-azidoADP) the membranes were incubated at 37 °C for 15 min in the presence of 1 mM ATP. At the end of the pretreatment IAAP (10 nM) was added to each of the three aliquots, and the mixture was incubated at 21−23 °C for 5 min and then photo-cross-linked, immunoprecipitated, and electrophoresed as described in the Experimental Procedures. The IAAP incorporated into the Pgp band was quantified as described above at the following steps: step 1, IAAP binding in the absence of any treatment; step 2, IAAP binding following Vi-induced trapping of 8-azidoADP; and step 3, IAAP binding after incubating Pgp with 1 mM ATP for 15 min at 37 °C following release of 8-azidoADP trapped during the first ATP hydrolysis. In (B) and (C) the empty bars represent wild-type Pgp and the filled bars the E556Q Pgp.

event (Figure 2A, panel I), the mutants E556Q and E1201Q exhibit drastically reduced Vi-induced [α-^{32}P]-8-azidoADP trapping (<10% compared to wild-type Pgp). These observations suggest that the defect in the mutants (E556Q and E1201Q) arises from their inability to initiate a second ATP hydrolysis event after the release of the nucleoside diphosphate. This raises the question, is it the nucleotide binding or the second nucleotide hydrolysis event following ADP release that is impaired? To address this question, we monitored [α-^{32}P]-8-azidoATP binding at 4 °C to wild-type and E556Q Pgp before and after trapping with 8-azidoADP and Vi (Figure 2B). We then washed off excess 8-azidoATP and Vi by centrifugation and incubated the samples at 37 °C for 15 min to release the 8-azidoADP, brought the

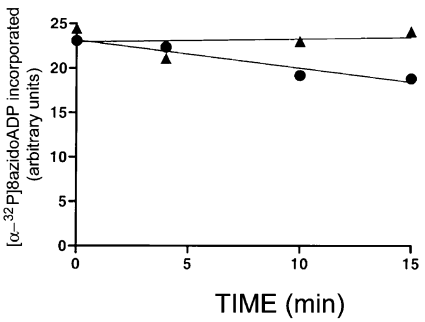

FIGURE 3: Time course of release of [α-^{32}P]-8-azidoADP in the double (E556/1201Q) mutant Pgp in the presence and absence of Vi. Crude membranes were incubated with 50 μM [α-^{32}P]-8-azidoATP (2 μCi/nmol) and in the presence and absence of 0.25 mM Vi in the ATPase assay buffer for 10 min at 37 °C. The time course of release of trapped [α-^{32}P]-8-azidoADP from the E556/1201Q Pgp mutant was then followed as described in the legend to Figure 2A, panel II, in the presence (▲) and absence (●) of 0.25 mM Vi.

samples to 4 °C, and reassessed the photo-cross-linking of [α-^{32}P]-8-azidoATP to Pgp under nonhydrolysis conditions. Both the wild-type and mutant E556Q Pgp show a marked decrease in the incorporation of [α-^{32}P]-8-azidoATP into Pgp in the Vi-trapped state (Figure 2B, step 2), consistent with our previous work (*23*), which is restored to normal levels after dissociation of 8-azidoADP (Figure 2B, step 3). Thus, the binding of nucleotide is normal, and it is the second ATP hydrolysis event per se that appears to be impaired in the mutant E556Q. Similar results were also obtained with the E1201Q mutant Pgp (data not shown).

How do these changes at the ATP site of Pgp affect drug substrate binding? We reported previously that binding of the Pgp photoaffinity substrate analogue IAAP is reduced by >90% in a transition state and, following ADP release, is recovered by incubating Pgp under conditions that permit ATP hydrolysis (*22*). As shown in Figure 2C (step 2), binding of IAAP to wild-type Pgp is decreased when the membranes are incubated with 8-azidoATP and Vi, and this is recovered (Figure 2C, step 3) after the second ATP hydrolysis event when the membranes are incubated at 37 °C with fresh ATP (1 mM) for 10 min (also see refs *22* and *23*). The mutant (E556Q) Pgp also shows reduced binding of IAAP in the Vi-trapped state. However, in contrast to wild-type protein, IAAP binding to the E556Q mutant Pgp is not recovered after incubation with 1 mM ATP, under hydrolysis conditions (Figure 2C, steps 2 and 3).

The Pgp Double Mutant, E556/1201Q, Does Not Release the Trapped Nucleotide. The experiment in Figure 1C shows that the double mutant E556/1201Q Pgp is able to trap [α-^{32}P]-8-azidoADP to the same extent in the absence or presence of Vi, a phenomenon that is distinct from that exhibited by wild-type Pgp or the single mutants (E556Q and E1201Q). We compared the time course of nucleotide trapping in the absence and presence of Vi for the Pgp double mutant E556/1201Q and determined that the rate of trapping of 8-azidoADP is comparable in the absence or presence of Vi (data not shown). However, unlike the wild type and E556Q or E1201Q mutant, the double mutant failed to release [α-^{32}P]-8-azidoADP trapped in both the presence and absence of Vi (compare Figure 2A, panel II, and Figure 3).

Characterization of Nucleotide Trapping in the Pgp Double Mutant E556/1201Q. The Vi-trapped transition state of Pgp (Pgp·MgADP·Vi) has been extensively exploited to understand the catalytic cycle of Pgp (*22*, *23*, *30*, *41*−*45*). Previous studies have clearly demonstrated that the trapped moiety is always the nucleoside diphosphate (*39*, *42*, *46*). This is also consistent with the mechanistic basis of the inhibition of ATPase activity of Pgp by Vi (*47*). As the Pgp double mutant E556/1201Q shows trapping of nucleotide even in the absence of Vi, it is not clear whether the trapped nucleotide is ATP or ADP. To address this issue, we incubated the double mutant E556/1201Q in the absence and presence of Vi with either [α-^{32}P]-8-azidoATP or [γ-^{32}P]-8-azidoATP. If the "trapped" state is reached following ATP hydrolysis, the γ-^{32}P will be cleaved, and there would be no radioactive signal following photo-cross-linking in the Pgp band. A complicating factor in the interpretation of results using [γ-^{32}P]-8-azidoATP is the fact that kinase-mediated phosphorylation would also transfer the γ-^{32}P to the Pgp. This can, however, be experimentally corrected by using a control sample that is not photo-cross-linked. The autoradiogram depicted in Figure 4A (lanes labeled as α) demonstrates that there is a ^{32}P signal associated with the Pgp band when the mutant Pgp (E556/1201Q) is incubated with [α-^{32}P]-8-azidoATP at 37 °C, in either the presence or absence of Vi which is not detected when [γ-^{32}P]-8-azidoATP is used in lieu of the α-^{32}P (lanes labeled as γ). There is no detectable kinase-mediated transfer of the γ-^{32}P as evidenced by the lack of a detectable signal in the absence of UV cross-linking, and thus one can conclude that the trapped nucleotide is the 8-azidoADP. Similarly, no γ-^{32}P-labeled band is visible with the wild-type Pgp, where it has previously been demonstrated using several different approaches that it is always the nucleoside diphosphate that is trapped (*39*, *42*, *46*). The binding of [γ-^{32}P]-8-azidoATP (obtained by incubation at 4 °C, when measurable ATP hydrolysis does not occur) is normal in wild-type and mutant Pgps (data not given).

We recently reported (*39*) that Vi-induced trapping of [α-^{32}P]-8-azidoADP, under nonhydrolysis conditions (i.e., when Pgp is incubated with [α-^{32}P]-8-azidoADP in the presence of Vi), is temperature-dependent with an activation energy of 152 kJ/mol. We observed that the Vi-induced trapping of [α-^{32}P]-8-azidoADP is considerably enhanced at 37 °C compared to that at 23 °C in both the wild-type and double mutant (E556/1201Q) Pgp. Moreover, the incorporation of [α-^{32}P]-8-azidoADP in both the absence and presence of Vi is similarly increased at 37 °C in the mutant Pgp (Figure 4B).

It has previously been demonstrated that drug substrates of Pgp stimulate both steady-state ATP hydrolysis (*34*, *36*, *48*−*52*) and Vi-induced [α-^{32}P]-8-azidoADP trapping (*36*, *52*, *53*). When [α-^{32}P]-8-azidoATP at low concentration (5 μM) was used, both the hydrolysis and subsequent Vi-induced trapping of nucleotide were found to be about 20 times slower than at saturating concentrations (*10*). This appreciably slower hydrolysis rate allows one to demonstrate the drug-stimulated trapping of [α-^{32}P]-8-azidoADP more convincingly. Thus, while studying the effect of verapamil (50 μM) on the Vi-induced trapping, we used 5 μM [α-^{32}P]-8-azidoATP instead of the 50 μM routinely used in trapping experiments. The results of this experiment depicted in Figure 4C show that the drug substrate verapamil stimulates Vi-induced trapping (∼2−2.5-fold) by wild-type Pgp. Similarly,

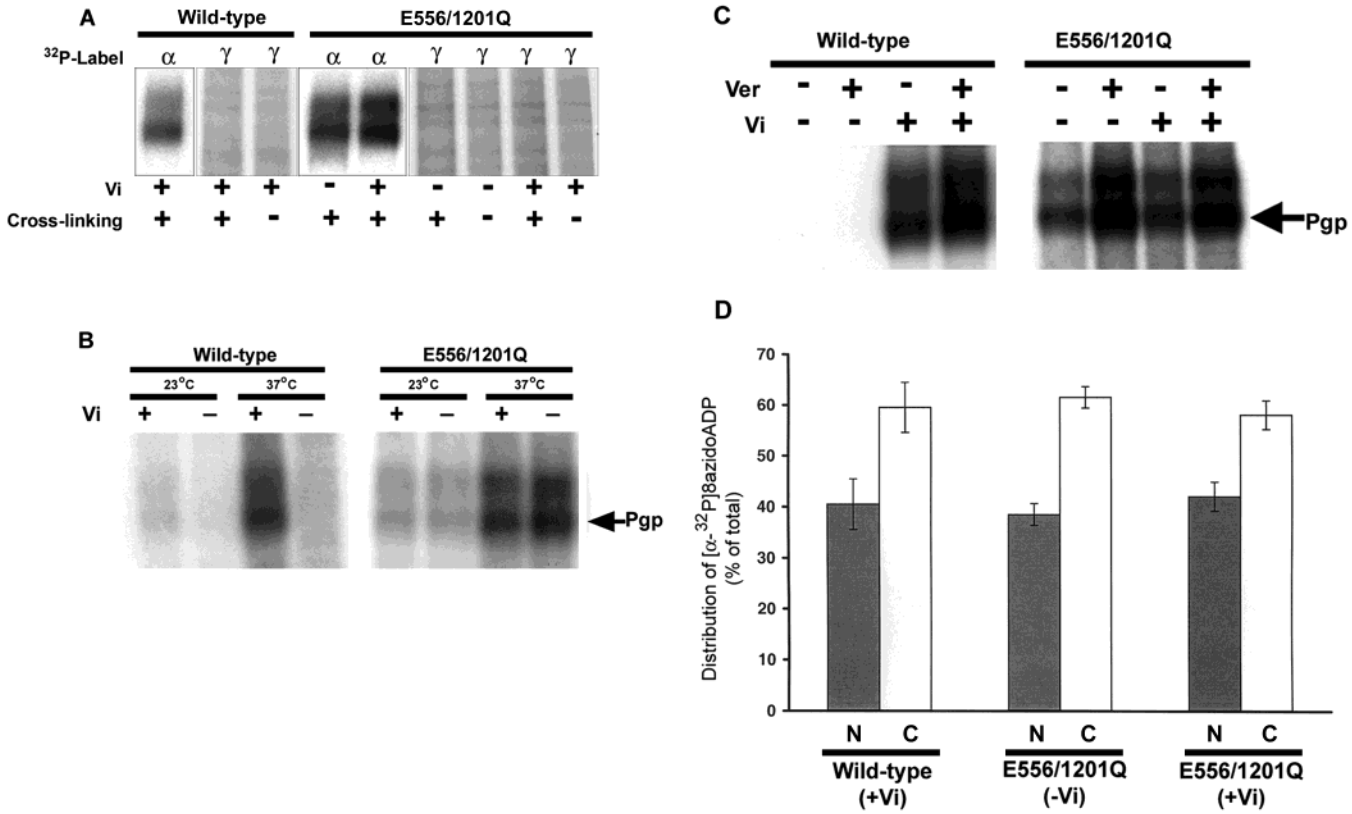

FIGURE 4: Determination of the nature of the trapped nucleotide in the Pgp double mutant E556/1201Q. (A) Vi-induced trapping in wild-type and the double mutant E556/1201Q Pgp by incubating with [α-^{32}P]-8-azidoATP or [γ-^{32}P]-8-azidoATP. Crude membranes (60 μg of protein) prepared from HeLa cells infected−transfected with pTM1-*MDR*1 wild type or the pTM1-*MDR*1-E556/1201Q double mutant were incubated either with [α-^{32}P]-8-azidoATP or [γ-^{32}P]-8-azidoATP (50 μM, 0.5 μCi/nmol) and 0.25 mM Vi at 37 °C in the ATPase assay buffer for 10 min at 37 °C. The reaction was stopped by adding 10 mM ice-cold ATP, and samples were photo-cross-linked as described above. When [γ-^{32}P]-8-azidoATP was used, two samples were simultaneously processed: one was photo-cross-linked and the other was not photo-cross-linked to assess the contribution of kinase-mediated phosphorylation of Pgp. Samples were processed as described in the legend to Figure 1C. (B) Effect of temperature on the trapping of [α-^{32}P]-8-azidoADP under nonhydrolysis conditions in the wild-type and mutant E556/1201Q Pgp. Crude membranes (60 μg of protein) were incubated with [α-^{32}P]-8-azidoADP (50 μM, 2 μCi/nmol) in the absence or presence of 0.25 mM Vi in the ATPase assay buffer for 10 min at either 23 or 37 °C. The reaction samples were processed as described in the Experimental Procedures. (C) Stimulation of trapping of [α-^{32}P]-8-azidoADP in the wild-type and double mutant E556/1201Q Pgp by verapamil. Crude membranes (60 μg of protein) were incubated with [α-^{32}P]-8-azidoATP (5 μM, 10 μCi/nmol) in the ATPase assay buffer for 5 min at 37 °C in either the absence or presence of 0.25 mM Vi. Experiments were performed in duplicate; one sample was treated with 50 μM verapamil at 37 °C for 5 min prior to the addition of Vi and [α-^{32}P]-8-azidoATP while the other was incubated with an equal volume of Me_2SO. The other details as given in the legend to Figure 1C. (D) Distribution of the trapped [α-^{32}P]-8-azidoADP between the N- and the C-terminal ATP sites of the wild-type and the double mutant E556/1201Q Pgp. Pgp crude membranes (60 μg of protein) were incubated with 50 μM [α-^{32}P]-8-azidoATP (2 μCi/nmol) in the ATPase assay buffer for 10 min at 37 °C. The mutant, E556/1201Q, Pgp was incubated in both the absence or presence of 0.25 mM Vi whereas the wild-type Pgp was incubated only in the presence of 0.25 mM Vi. The reaction was stopped by adding 10 mM ice-cold ATP, and samples were photo-cross-linked as described in the Experimental Procedures. Samples were then treated with TPCK-treated trypsin (6 μg per sample) for 5 min at 37 °C (*12*). The reaction was stopped by adding 30 μg of trypsin inhibitor. Control and trypsin-treated samples were processed as described in the Experimental Procedures. The graph shows the distribution of [α-^{32}P]-8-azidoADP in the N- (filled bars) and the C- (empty bars) halves of the wild-type and mutant E556/1201Q Pgp, which are labeled as N and C on the graph. The error bars represent the standard deviation ($n = 3$). [α-^{32}P]-8-AzidoADP was incorporated into the mutant Pgp in both the presence (+Vi) and absence of Vi (−Vi) whereas in wild-type Pgp, it was incorporated only in the presence of Vi (+Vi) (see Figure 1C).

nucleotide trapping in both the absence or presence of Vi is stimulated by verapamil in the double mutant (E556/1201Q) protein. Therefore, the mutant Pgp, E556/1201Q, supports at least a single ATP hydrolysis event, and the trapped state obtained in the absence or presence of Vi appears to be similar. To determine whether the N-terminal or the C-terminal ATP site was preferentially utilized in the double mutant, the distribution of [α-^{32}P]-8-azidoADP in both halves (each half containing one ATP site) of the mutant Pgp was measured following mild trypsinization. With the wild-type protein at saturating concentrations of [α-^{32}P]-8-azidoATP in the presence of Vi, [α-^{32}P]-8-azidoADP was trapped almost equally into both the N- and the C-halves of wild-type Pgp (*39*, *54*, *55*). We found that the distribution of [α-^{32}P]-8-azidoADP in the N- and the C-terminal halves of the double mutant E556/1201Q Pgp is comparable to that observed with wild-type protein (Figure 4D), and there was no difference in the distribution of [α-^{32}P]-8-azidoADP whether the nucleotide was trapped in the absence or presence or Vi. Similarly, the distribution of the trapped [α-^{32}P]-8-azidoADP was also found to be more or less equal in both the N- and the C-terminal ATP sites in the single mutant Pgps, E556Q and E1201Q (data not given).

Since E556 and E1201 are located next to D555 and D1200, which are essential for Mg^{2+} binding (*11*, *12*), it would be interesting to determine whether mutations of the

glutamates at positions 556 and 1201 affect interactions with divalent cations. We find that the requirement for cations, during trapping of [α-^{32}P]-8-azidoADP, is similar for the wild-type and mutant Pgps E556Q, E1201Q, and E556/1201Q. There is a negligible level of trapping in the presence of EDTA (absence of cations), and the divalent cations Mg^{2+} and Mn^{2+} are critical for trapping to occur. Additionally, the monovalent cation Na^+ up to a concentration of 150 mM does not permit trapping of [α-^{32}P]-8-azidoADP in either the wild-type or any of the mutant Pgps in the absence of Mg^{2+} (data not shown). Thus, though the conserved residues E556 and E1201 are adjacent to D555 and D1200 which are involved in the coordination of Mg^{2+} (*11*, *12*), substitutions at positions 556 and 1201 or both do not affect cation specificity (data not shown).

Are the Transition States Formed by the Pgp Mutant E556/1201Q in the Absence and Presence of Vi Comparable? The results described above indicate that the double mutant E556/1201Q traps [α-^{32}P]-8-azidoADP to form a transition state similar to that of wild-type Pgp. However, as the mutant Pgp generates the "transition state" even in the absence of Vi, it would be important to understand the properties of this complex. Previous work has established that beryllium fluoride (BeF_x) inhibits the Pgp ATPase activity by generating a transition state, Pgp·MgADP·BeF_x, which is distinct from the Pgp·MgADP·Vi transition state (*46*). The most significant difference between these two transition states is that sodium pyrophosphate (PP_i) protects against the BeF_x-induced inhibition of ATPase activity, and trapping of ADP but has no effect on Vi-induced inhibition of activity or trapping of nucleotide (*46*). On the basis of these results and the crystal structure of the myosin·MgADP·BeF_x complex, the transition state generated by using BeF_x has been termed the "prehydrolysis state" (*56*). We exploited this system to understand whether the trapping of nucleotide by the Pgp double mutant (E556/1201Q) in the absence of Vi is equivalent to the Vi- or BeF_x-induced transition state conformation of wild-type Pgp. We demonstrate in Figure 5A (left panel) that in wild-type Pgp the Vi-induced trapping is not significantly inhibited by PP_i (5mM) whereas BeF_x-induced trapping is inhibited by >90%. However, we observe that for the Pgp double mutant, E556/1201Q, trapping under all three conditions (absence of any P_i analogue, presence of Vi or BeF_x) is unaffected by PP_i (Figure 5A, right panel). This would suggest that following ATP hydrolysis the double mutant spontaneously traps the resultant nucleoside diphosphate in a transition state that, by this test, does not differ from the one generated in the presence of Vi in wild-type Pgp.

The Double (E556/1201Q) Mutant Pgp in the Trapped State Generated in the Absence of Vi Exhibits Reduced Binding of the Drug Substrate, IAAP. Our recent studies (*22*, *23*, *35*) show that though binding of nucleotide to Pgp per se does not affect its interactions with the substrate, the Vi-trapped conformation of Pgp binds substrates with drastically reduced affinity (also see Figure 2C, step 2). Consistent with these studies, Figure 5B (left panel) shows that the binding of IAAP to wild-type Pgp is unaffected by incubating crude membranes with ATP alone at 37 °C prior to incubation with IAAP. However, the binding of IAAP is considerably diminished when the crude membranes are incubated with ATP in the presence of Vi. The mutant Pgp E556/1201Q, on the other hand, shows reduced binding of IAAP when incubated with ATP alone or in the presence of ATP and Vi prior to labeling with IAAP (Figure 5B, right panel). This experiment further supports the view that the transition state generated by the Pgp double mutant E556/1201Q in the absence of Vi has a conformation similar to that of the Pgp·ADP·Vi-trapped state of the wild-type protein.

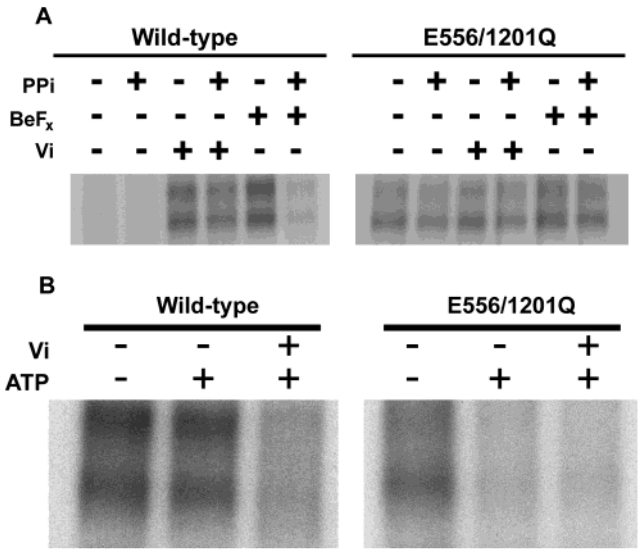

FIGURE 5: Characterization of [α-^{32}P]-8-azidoADP trapping by the double mutant E556/1201Q Pgp in the absence and presence of Vi. (A) Vanadate- and beryllium fluoride-induced trapping of [α-^{32}P]-8-azidoADP in wild-type Pgp and the double mutant Pgp E556/1201Q. Crude membranes were incubated with [α-^{32}P]-8-azidoATP (50 μM, 2 μCi/nmol) in the ATPase assay buffer for 10 min at 37°C under the following conditions: control, no additions; 5 mM sodium pyrophosphate (PP_i); 0.25 mM Vi; 0.25 mM Vi + 5 mM PP_i, 0.6 mM $BeSO_4$ + 2.5 mM NaF (BeF_x); 0.6 mM $BeSO_4$ + 2.5 mM NaF + 5 mM PP_i. The samples were processed as described in the legend to Figure 1C. (B) Effect of ATP and ATP + Vi on the photoaffinity labeling of the wild-type and double mutant E556/1201Q Pgp with IAAP. Crude membranes were incubated in the ATPase assay buffer for 10 min at 37 °C under the following conditions: (i) control, no additions; (ii) 1 mM ATP; and (iii) 1 mM ATP + 0.25 mM Vi. The reaction was stopped by transferring the samples to ice, and the samples were washed by centrifugation at 300000*g* for 15 min at 4 °C. IAAP (10 nM) was added to each of these aliquots, and the mixture was incubated at 23 °C for 5 min and then photo-cross-linked, immunoprecipitated, and electrophoresed as described in the Experimental Procedures.

Intact HeLa Cells Expressing the Mutant (E556Q, E1201Q, and E556/1201Q) Pgps Show Reduced Binding of the Drug Substrate, IAAP. The experiments described above have been conducted using crude membrane preparations of HeLa cells overexpressing the wild-type and mutant Pgps. The crude membranes are prepared and stored in media, which contain no ATP. Pgp exhibits a very low affinity for ATP (K_m of approximately 0.3−1 mM), and it is unlikely that there would be any ATP associated with the membrane preparations. Intact cells, on the other hand, would contain physiological levels of intracellular ATP. Thus the mutant Pgps in these cells would, following an initial ATP hydrolysis event, be trapped in a conformation(s) that does not favor the binding of the drug substrate, IAAP (see Figure 2C, step 2, and Figure 5B, right panel). To test this hypothesis, intact HeLa cells overexpressing the wild-type or the mutant (E556Q, E1201Q, E556/1201Q) Pgps were incubated with IAAP and photo-cross-linked. The photolabeled Pgp was immunoprecipitated,

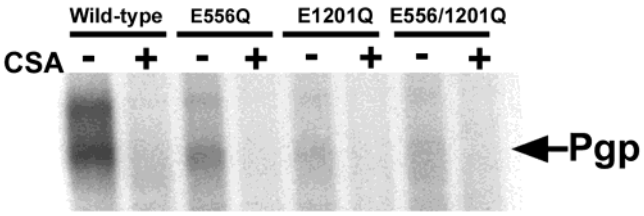

FIGURE 6: Photoaffinity labeling of wild-type and mutant (E556Q, E1201Q, and E556/1201Q) Pgps in intact HeLa cells. Infected−transfected HeLa cells (500000 cells resuspended in IMDM medium + 5% FBS) were labeled with IAAP (10 nM) in either the absence or presence of 5 μM cyclosporin A (CSA) and photo-cross-linked as described in the Experimental Procedures. The experimental conditions are given on the autoradiogram.

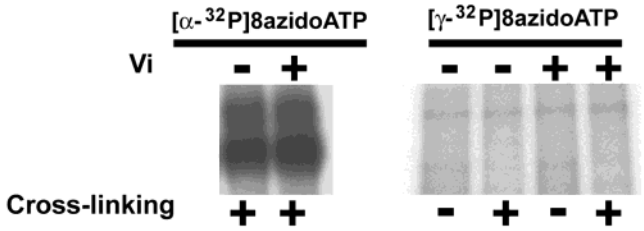

FIGURE 7: Determination of the nature of the trapped nucleotide in the Pgp double mutant, E556/1201A, in the presence and absence of Vi using [α-^{32}P]-8-azidoATP or [γ-^{32}P]-8-azidoATP. Crude membranes (60 μg of protein) were incubated in the ATPase assay buffer for 10 min at 37 °C under the following conditions: [α-^{32}P]-8-azidoATP (50 μM, 0.5 μCi/nmol); [α-^{32}P]-8-azidoATP (50 μM, 0.5 μCi/nmol) + 0.25 mM Vi; [γ-^{32}P]-8-azidoATP (50 μM, 0.5 μCi/nmol); [γ-^{32}P]-8-azidoATP (50 μM, 0.5 μCi/nmol) + 0.25 mM Vi. The reactions were stopped, and samples were processed as described in the legends to Figures 1C and 4A.

and Figure 6 demonstrates that wild-type Pgp shows binding of IAAP (which is sensitive to the Pgp modulator cyclosporin A), whereas the mutant Pgps (E556Q, E1201Q, and E556/1201Q) show significantly reduced binding of IAAP. These data indicate that in intact cells the mutant proteins are trapped into an intermediate conformation of the catalytic cycle that prohibits the completion of one cycle and, consequently, the initiation of the next cycle.

The Pgp Double Mutant E556/1201A Also Exhibits [α-^{32}P]-8-AzidoADP Trapping. The experiments described above indicate that substitution of E556, in the N-terminal ATP site, or its equivalent residue (E1201) with Q, in the C-terminal ATP site, or both (E556Q, E1201Q, or E556/1201Q) does not have any significant effect on the cleavage of $\beta-\gamma$-phosphate bond of ATP per se but affects subsequent steps in the catalytic cycle of Pgp. This is particularly significant because E556 or E1201 (and its homologues in other ABC transporters as well as other ATPases) has been implicated as the catalytic carboxylate (*10*, *13*, *57*). To assess whether a Glu to Ala substitution will affect the cleavage of the $\beta-\gamma$-phosphate bond of ATP, we generated the mutant Pgps where Glu was replaced with Ala (E556A, E1201A, and E556/1201A). Characterization of these mutants showed that (1) they exhibited comparable cell surface expression, (2) similar to E → Q (see Figure 1A), E → A substitutions also resulted in loss of transport activity, and (3) the single mutants E556A and E1201A, similar to E556Q and E1201Q (see Figure 1C), showed some trapping of [α-^{32}P]-8-azidoADP in the absence of Vi, which was enhanced in the presence of 0.25 mM Vi (data not shown). To determine the nature of the nucleotide trapped in the double mutant, E556/1201A, the Vi-induced trapping was carried out using [α-^{32}P]-8-azidoATP as well as [γ-^{32}P]-8-azidoATP. The double mutant E556/1201A shows [α-^{32}P]-8-azidoADP trapping in both the absence and presence of Vi (Figure 7, left panel), which is comparable to the double mutant E556/1201Q (Figure 4A). Furthermore, there was no discernible band when the double mutant E556/1201A was incubated with [γ-^{32}P]-8-azidoATP in either the absence or presence of Vi (Figure 7, right panel). The results from this experiment suggest that the glutamate residues at positions 556 and 1201 in human Pgp may not be critical for the cleavage of the $\beta-\gamma$ bond of ATP per se by Pgp. Moreover, the double mutant E556/1201A when incubated with ATP in either the absence or presence of Vi at 37 °C showed reduced affinity for IAAP (data not given), which is comparable to the finding with the double mutant E556/1201Q (Figure 5B). Additionally, the trapping of [α-^{32}P]-8-azidoADP in the absence of Vi by the double mutant E556/1201A is not sensitive to PP_i (data not given), similar to the observation with the E556/1201QA double mutant (Figure 5A). Thus, Pgp mutants with substitution of E556/1201 with either Q or A exhibit similar properties, and these data indicate that E556 and E1201 residues do not function as catalytic carboxylates.

DISCUSSION

The superfamily of ABC transport proteins represents numerous therapeutic targets (*1*). Pgp is one of the most extensively studied ABC transport proteins as its reversal could contribute significantly to overcoming clinical MDR and it is a good model system to understand ABC transporters in general. Building upon the model proposed by Senior and associates (*47*), we have in recent years elucidated the catalytic cycle of Pgp in considerable detail (*22*, *23*, *36*, *39*, *40*). The essential feature of our model is that ATP hydrolysis results in a dramatic conformational change where the affinity of both the substrate and the nucleotide for Pgp is reduced >30-fold and the hydrolysis of an additional molecule of nucleotide is obligatory to "reset" the molecule. In this study we investigated the role of highly conserved glutamate residues at positions 556 and 1201 in human Pgp to determine the following: (i) Is the presence of glutamate at these positions mandatory for cleaving the $\beta-\gamma$-phosphate bond of ATP? (ii) Does substitution of these residues with glutamine or alanine affect human Pgp function, and if so, what is the mechanistic basis?

Human Pgp mutants of the conserved glutamate residue in the Walker B region (E556Q, E556A, E1201Q, E1201A, E556/1201Q, and E556/1201A) showed cell surface expression levels comparable to that of the wild-type protein, but the transport function was abrogated in all of the mutant Pgps (Figure 1A,B; data for E556A, E1201A, and E556/1201A not shown). Moreover, our results are in agreement with the findings in Mdr3 that show that replacement of Glu 556 or Glu 1201 with Gln causes a loss of steady-state ATPase activity but permits Vi-induced trapping of [α-^{32}P]-8-azidoADP (Figure 1C and ref *10*). These observations suggest that these Pgp mutants can catalyze a single ATP hydrolysis event. But, whether the glutamate residue is necessary for ATP hydrolysis cannot be addressed on the basis of this evidence as only one of the two ATP sites is modified and there is the confounding influence of the

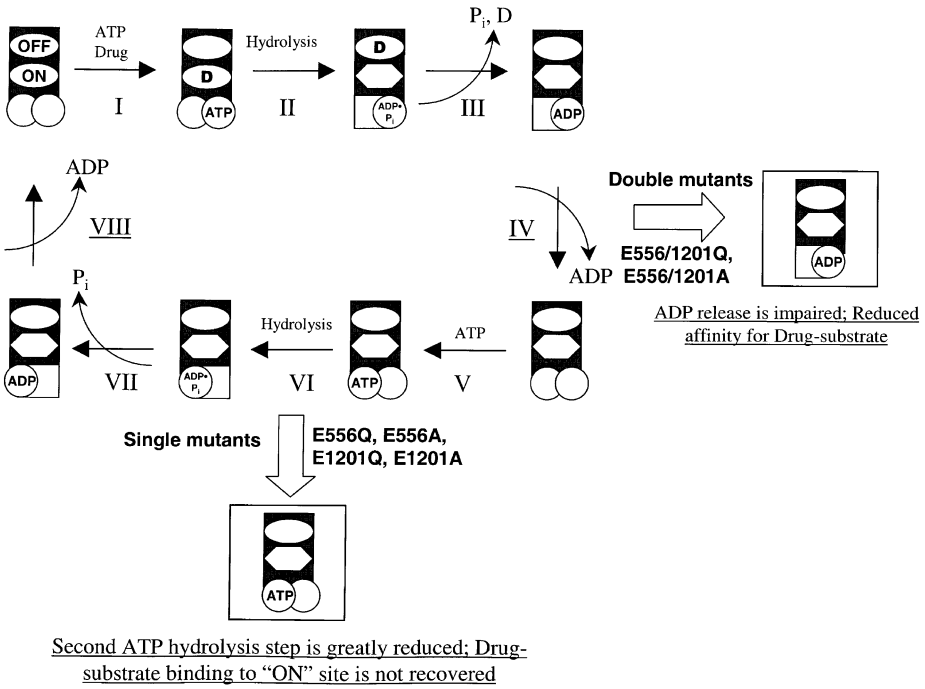

FIGURE 8: Effect of the substitution of E556, E1201, and E556/E1201 with Q or A on the catalytic cycle of ATP hydrolysis by Pgp. The scheme for the catalytic cycle of Pgp has been described earlier (*23*). The ellipses represent the substrate binding sites; the "ON" and the "OFF" site. The hexagon portrays the ON site with reduced affinity for the drug. The circles represent the ATP sites. The empty square represents the ATP site with reduced affinity for nucleotide. Step I: Substrate binds to the high-affinity ON site of Pgp, and ATP binds to either of the two ATP sites. Step II: ATP is hydrolyzed, and the drug is moved to the lower affinity OFF site. Step III: P_i is released, and the drug is extruded from Pgp at this step. Step IV: The ADP and Vi dissociate from the complex, the ATP sites revert to the high-affinity state, but affinity for the drug substrate continues to be low. Step V: Following disassociation of the ADP in step IV, an additional molecule of ATP binds to the alternate ATP site. Step VI: ATP is hydrolyzed. Step VII: P_i is released. Step VIII: The disassociation of ADP allows the conformation of Pgp to be restored to its original state (step I) to initiate the next cycle (*23*). Since the double mutants E556/1201Q or E566/1201A are impaired in the release of ADP (see Figure 3; data for E556/1201A not shown), the catalytic cycle is possibly terminated at step IV. How the ADP release is impaired is currently not known. It is likely that the signaling from the functional alternate site is required for the ADP release from the catalytic site. The single mutants (E556Q, E556A, E1201Q, and E1201A), on the other hand, show normal release of ADP and can bind ATP during next step but exhibit greatly reduced ability to hydrolyze it (see Figure 2A, panels I−III; data with E556A and E1201A not shown). In these mutants thus, the catalytic cycle is impaired at step VI. It is important to note that though the single and the double mutants are impaired at different steps, these mutants are unable to complete the catalytic cycle, resulting in loss of steady-state substrate-stimulated ATP hydrolysis and drug transport activities.

unmodified ATP site. We therefore studied the double mutant, where residues in both ATP sites are simultaneously modified (E556/1201Q or E556/1201A).

There is a general consensus that when Vi-induced trapping of nucleotide is observed, at least one ATP hydrolysis event (or "half-cycle") has necessarily occurred (*39*, *42*, *46*). However, as the double mutant (E556/1201Q) incorporates nucleotide in both the absence or presence of Vi (Figure 1C), we cannot assume that ATP hydrolysis has necessarily occurred and that the mutant Pgp does not merely trap ATP tenaciously. We therefore used several approaches to address this issue. We compared the trapping of the nucleotide by using both the α-^{32}P- and the γ-^{32}P-labeled 8-azidoATP and found that when [γ-^{32}P]-8-azidoATP was used, there was no ^{32}P signal associated with the Pgp band (Figures 4A and 7), though in nucleotide binding experiments carried out at 4 °C, both α-^{32}P- and γ-^{32}P-labeled 8-azidoATP were incorporated equally into the Pgp protein (data not shown). This implies that in the Pgp double mutants E556/1201Q and E556/1201A the trapped moiety is the 8-azidoADP in both the absence and presence of Vi. Moreover, the effect of temperature on the trapping of 8-azidoADP in the double mutants (Figure 4B) is consistent with the energetics of trapping rather than binding (see ref *39* for details). Finally, we determined that the nucleotide trapping in both the absence or presence of Vi is stimulated by verapamil in the E556/1201Q Pgp (Figure 4C). As drug substrates have no effect on the binding per se of [α-^{32}P]-8-azidoATP to Pgp (*23*), the stimulation of nucleotide trapping strongly indicates the occurrence of ATP hydrolysis.

It could be argued that when the conserved glutamates were substituted with glutamines, these could also activate a water molecule to attack the γ-phosphate of ATP. However, the double mutant E556/1201A also shows a phenotype similar to the mutant E556/1201Q, in that it traps the nucleoside diphosphate in both the absence and presence of Vi (Figure 7). These results thus suggest that activation of a water molecule by the glutamate residues at positions 556 and 1201 in human Pgp is not obligatory for the cleavage of the bond between the β- and γ-phosphates of ATP. It has been argued largely on the basis of the fact that the mutant E171Q (equivalent to E556 or E1201 of human Pgp) in MJ0796 of *Methanococcus jannaschii* had undetectable steady-state ATPase activity that these residues are catalytic carboxylates in ABC transport proteins (*58*, *59*). This observation in itself is consistent with our finding with human Pgp and that of Urbatsch et al. (*10*) with mouse Pgp. We demonstrate here a single ATP hydrolysis event using Vi -trapping to generate the transition state conformation of Pgp. Similar experiments with MJ0796 in the presence of the

membrane subunits to form a "transport-competent complex" to generate the Vi-induced transition state conformation (*60*−*62*) will be required to determine whether the residue E171 is a catalytic carboxylate. Other candidates for activation of the attacking water for ATP hydrolysis in Pgp, Q471 and Q1114, in NBDs of mouse Mdr3 have also been shown not to play such a role (*21*). It is thus plausible that the nucleotide itself may be the base and that water transfers its proton directly to the γ-phosphate as in the case in myosin (for review see ref *63*). What then is the role of the residues E556 and E1201 in Pgp?

The mutations studied here provide additional evidence for the occurrence of two ATP hydrolysis events in a single catalytic cycle of Pgp (*22*, *23*), and their mechanistic basis is illustrated in Figure 8. The Pgp mutants E556Q and E1201Q might be expected to be fully functional as both NBDs hydrolyze ATP and release ADP to the same extent as wild-type Pgp. However, the finding that the mutation in a single ATP site (either the N- or the C-terminal) obstructs the ATP hydrolysis after a single ATP hydrolysis event suggests that the sites are used in an alternate fashion as proposed earlier (*47*). Thus, E556 or E1201 may be critical in recruiting the second site after the ATP hydrolysis event at the first site (see refs *22* and *23* for an explanation of the two ATP hydrolysis events). The model proposed in Figure 8 would predict that in intact HeLa cells, due to the availability of intracellular ATP, the single Pgp mutants would be locked in the conformation at step V and the Pgp double mutants would be in the conformation at step IV (Figure 8). Our earlier work has demonstrated that reduced affinity for IAAP would be a diagnostic feature of the conformation of Pgp at both of these steps in the catalytic cycle (*22*, *23*). Indeed, the data given in Figure 6 indicate that the binding of IAAP is significantly reduced in the intact cells expressing the mutant Pgps (E556Q, E1201Q, and E556/1201Q, respectively) compared to wild-type Pgp.

In addition to providing insights into the catalytic mechanism of Pgp, the double mutant Pgps (E556/1201Q and E556/1201A) provide a means of trapping Pgp in the transition state in the absence of Vi or BeF_x, and we present evidence that the 8-azidoADP-trapped state obtained in the double mutants is comparable to the Vi transition state of wild-type Pgp (see Figures 4, 5B, and 7). The Vi-induced transition state conformation of Pgp has been a very useful experimental tool in characterizing the catalytic cycle of Pgp (for review see ref *64*). Finally, we distinguish the nature of the transition state that the mutant (E556/1201Q) Pgp attains in the absence of Vi. Besides the Vi-trapped state of Pgp another well-characterized transition state is that induced by BeF_x, and these transition states can be biochemically distinguished from each other. Sodium pyrophosphate (PP_i) inhibits BeF_x-induced trapping but has no effect on Vi-induced trapping (*46*). However, the [α-^{32}P]-8-azidoADP trapping by the Pgp double mutant E556/1201Q is not inhibited by PP_i (Figure 5A), suggesting that the transition state formed by the mutant Pgp, E556/1201Q, closely mimics the Vi-induced Pgp·MgADP·Vi transition state of wild-type Pgp.

This study suggests that residues E556 and E1201 in human Pgp are critical for the second ATP hydrolysis event which we have previously demonstrated is associated with resetting the Pgp molecule following the transport of drug (*22*, *23*). These residues are likely to be a part of the switch region of ABC transport proteins deemed to be involved in transmission of interdomain signals from the substrate-binding sites via the D loop and the ABC signature region (*25*, *26*). If this were the case, one would expect that mutations in some of the conserved residues in the D loop (*10*) or the ABC signature region (*65*) might also exhibit similar behavior. The ability of the double mutants (E556/1201Q or E556/1201A) to generate the transition state in the absence of Vi or BeF_x is quite unique and has not yet been reported for any other ABC transporter. It is, however, unclear how the double mutants (E556/1201Q and E556/1201A) generate the transition state intermediate by trapping the [α-^{32}P]-8-azidoADP in the absence of Vi or BeF_x. The resolution of the structure of the Pgp molecule and additional work on site-directed mutagenesis of residues in ATP sites should provide insight into the formation of the transition state in the double mutants.

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