The transport cycle of ABC transporters in general and P-glycoprotein in particular has been extensively studied, but the molecular mechanism remains controversial. We identify stable reaction intermediates in the progression of the P-glycoprotein-mediated ATPase reaction equivalent to the enzyme-substrate (ES, P-glycoprotein-ATP) and enzyme-product (EP, P-glycoprotein-ADP-P) reaction intermediates. These have been characterized using the photoaffinity analog 8-azido-[α-32P]ATP or [α-32P]ATP, in which a cross-linking step is not involved. Similar results were obtained when 8-azido-[α-32P]ATP was used. The reaction intermediates were characterized based on their kinetic properties and the nature (triphosphate/diphosphate) of the trapped nucleotide. Using this defined framework and the Walker B E56Q/E120Q mutant that traps nucleotide in the absence of vanadate or beryllium fluoride, the high to low affinity switch in the transport substrate binding site can be attributed to the formation of the E-S reaction intermediate of the ATPase reaction. Importantly, the posthydrolysis EP state continues to have low affinity for substrate, suggesting that conformational changes that form the E-S complex are coupled to the conformational change at the transport substrate site to do mechanical work. Thus, the formation of E-S reaction intermediate during a single turnover of the catalytic cycle appears to provide the initial power stroke for movement of drug substrate from inner leaflet to outer leaflet of lipid bilayer. This novel approach allows transition state theory to elucidate the mechanism of P-glycoprotein and other ABC transporters and provides wider applications in testing cause-effect hypotheses in coupled systems.

The ATP-binding cassette (ABC) family of transport proteins is one of the largest families of proteins in living organisms (1, 2). Understanding how these proteins work is extremely important because of their central role in biology as well as the fact that many members of this group are directly implicated in human diseases (2). The transport cycle of ABC proteins in general and the MDR1 (ABCB1) gene product, P-glycoprotein (Pgp), often regarded as the prototypical ABC transporter, has received considerable attention (see Refs. 3–6 for reviews). The ABC transport proteins have discrete nucleotide binding domains (NBDs) and transport substrate binding sites (7), and it is generally accepted that transport of various substrates from simple ions to complex toxic compounds and proteins involves coupling the energy of ATP hydrolysis to mechanical movements at the transport substrate sites (3–5). However, the mechanism of transport is still poorly understood. The challenge has been to identify the causal events at the NBDs that drive conformational changes at the transport substrate sites in the transmembrane domains.

Experimental monitoring of the transformation of a high affinity transport substrate site to a low affinity site has been used by several groups (8–12) as a surrogate assay for the power stroke that moves the molecular cargo from the inward facing, high affinity site to an outward facing, low affinity site from which it is expelled. Concomitantly, numerous studies have demonstrated that ATP hydrolysis provides the energy for transport in ABC transport proteins (4, 7, 13–15), clearly identified and characterized reaction intermediates of the ATPase reaction pathway could offer a well defined framework to study the conformational changes that drive transport.

The reaction intermediates of enzymatic reactions exist in transition states that have lifetimes in the range of 10−13 s and cannot be observed by physical or spectrometric methods (16). Enzymes can, however, be captured in their transition states by arresting the reaction intermediates of the chemical reaction at specific steps either by chemical modifications of the substrate or mutations in the enzyme. Numerous ATPases form pentacoordinate complexes with ADP and Vi, and many of these have been shown in crystal structures to represent transition states (for a review, see Ref. 17). The Vi-induced, ADP-trapped state
of Pgp has been well characterized (18, 19), and is believed to be comparable with the posthydrolysis, Pgp-ADP-P, reaction intermediate. Moreover, recent studies have shown that mutating the highly conserved glutamates (Glu556 and Glu1201) in human Pgp in the Walker B region results in a phenotype wherein the protein shows no transport function and minimal steady-state ATP hydrolysis but can occlude the nucleotide (20). One explanation for this has been that these conserved glutamates constitute the catalytic carboxylate; consequently, the mutant cannot cleave the bond between the β-P and the γ-P, and ATP is “trapped” (21, 22). If this were so, the double mutant (E556Q/E1201Q) may, in the presence of nucleotide, represent a prehydrolysis transition state of the Pgp-catalyzed ATP hydrolysis reaction. Such a view is consistent with several recent structures of the NBDs of ABC proteins that suggest that ATP acts as a molecular glue bringing together the two NBDs to form the ATP sandwich dimer. Moreover, in recent years, it has been suggested that the formation of the dimer may be coupled to mechanical movements in the transport substrate site. For example, it has been postulated that whereas ATP-driven dimerization provides the power-stroke for the pump, ATP hydrolysis resets the Pgp molecule (5).

In this study, we have further characterized and defined reaction intermediates of Pgp-mediated ATP hydrolysis based on their kinetics and the nature of the occluded nucleotide. We also show strong coupling between the NBDs and the transport substrate site(s) in purified and reconstituted wild-type and mutant (E556Q/E1201Q) Pgps. The conformational changes in Pgp that accompany transport were studied within the framework of the progression of the Pgp-mediated ATPase reaction. This study thus provides a general strategy to more precisely identify the driving force for the mechanical (conformational) changes that bring about initial drug substrate movement within the bilayer. We estimated the activation energies for the formation of the nucleotide triphosphate-trapped (prehydrolysis) state using the mutant (E556Q/E1201Q) Pgp as well as the nucleotide diphosphate-trapped (posthydrolysis) state. These results were obtained both by photocross-linking the trapped nucleotide using 8-azido-[α-32P]ATP and by directly estimating the occluded [α-32P]ATP/ADP. Moreover, we also estimated the activation energy for the reduced binding of the transport substrate, [125I]iodoarylazidoprazosin (125I]IAAP). Our results show that the conformational changes that transform the high affinity transport substrate binding site to a low affinity site occur during the formation of the Pgp-ATP (ES) reaction intermediate of the ATPase catalytic cycle but not independent of ATP hydrolysis.

**Experimental Procedures**

**Chemicals**—[125I]IAAP (2,200 Ci/mmol) and [α-32P]ATP (3,000 Ci/mmol) were obtained from PerkinElmer Life Sciences. 8-Azido-[α-32P]ATP (15–20 Ci/mmol), 8-azido-[α-32P]ADP (15–20 Ci/mmol), 8-azido-ATP, and 8-azido-ADP were purchased from Affinity Labeling Technologies, Inc. (Lexington, KY). The Pgp-specific monoclonal antibody C-219 was obtained from Fujirebio Diagnostics Inc. (Malvern, PA). All other chemicals were obtained from Sigma.

**Preparation of Crude Membranes from High Five Insect Cells Infected with Recombinant Baculovirus Carrying the Wild-type and Mutant Human MDR1 Gene**—High Five insect cells (Invitrogen) were infected with the recombinant baculovirus carrying the human MDR1 cDNA (either wild type or the mutant, E556Q/E1201Q) with a His6 tag at the C-terminal end as described (9). Crude membranes were prepared as described previously (9, 11).

**Purification and Reconstitution of Pgp**—Human Pgp from crude membranes of High Five insect cells was purified as described previously (9, 23). The crude membranes were solubilized with octyl-β-D-glucopyranoside (1.25%) in the presence of 20% glycerol and a lipid mixture (0.1%). Solubilized proteins were subjected to metal affinity chromatography (Talon resin; Clontech, Palo Alto, CA) in the presence of 0.95% octyl-β-D-glucopyranoside and 0.04% lipid; 80% purified Pgp was eluted with 200 mM imidazole. Pgp in the 200 mM imidazole fraction was then concentrated (Centriprep-50; Amicon, Beverly, MA) to 0.5–1.5 mg/ml and stored at −70 °C. Pgp was identified by immunoblot analysis using the monoclonal antibody C219 and quantified by the Amido Black protein estimation method as described previously (23). Purified Pgp was reconstituted into proteoliposomes by dialysis using a lipid/protein ratio of 6:1 as described (24, 25). We have previously experimentally determined that Pgp that is inserted in proteoliposomes is >95% in inside-out orientation (NBDs facing extravesicular space (25, 26)).

**Photocross-linking of 8-Azido[α-32P]ATP to Pgp**—Crude membranes of High Five insect cells (50–100 μg of protein) or purified and reconstituted protein (5–10 μg of protein) were incubated in the ATPase assay buffer (50 mM MES-Tris, pH 6.8, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, 2 mM dithiothreitol, 1 mM ouabain, and 10 mM MgCl2) containing 10 μM 8-azido-[α-32P]ATP (10 μCi/nmol) in the dark at 4 °C. The samples were irradiated with a UV lamp assembly (PGC Scientific, Gaithersburg, MD) fitted with two black light (self-filtering) UV-A long wave F15T8BLB tubes (365 nm) for 10 min on ice (4 °C). Ice-cold ATP (10 mM) was added to displace excess noncovalently bound 8-azido-[α-32P]ATP. After SDS-PAGE on a 7% NuPAGE gel, the gels were dried and exposed to Bio-Max MR film (Eastman Kodak Co.) at −70 °C for 12–24 h. The radioactivity incorporated into the Pgp band was quantified using the STORM 860 PhosphorImager system (Amersham Biosciences) and the software ImageQuant.

**Vanadate-induced 8-Azido[α-32P]ATP Trapping to Pgp**—The Pgp-8-azido-ADP-Vi reaction intermediate was generated as described previously (19). Crude membranes of High Five insect cells (100 μg) or purified and reconstituted protein (5–10 μg) were incubated in the ATP assay buffer (see above) containing 50 μM 8-azido-[α-32P]ATP (5 μCi/nmol) and 300 μM Vi in the dark at 37 °C for 5 min. The reaction was stopped by the addition of 10 mM ice-cold ATP and placing the samples immediately on ice. The trapped nucleotides were photocross-linked and electrophoresed, and the radioactivity incorporated in the Pgp band was quantified as described (19).

**Photoaffinity Labeling with [125I]IAAP**—The crude membranes of High Five insect cells (1.0 mg/ml) or purified and reconstituted Pgp (100 μg/ml) were incubated at room temper-
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Our use of photoaffinity analogs has been criticized (29) on the grounds that these measurements are not carried out under equilibrium conditions. To allay these concerns, we have estimated the kinetic and thermodynamic parameters using both the photoaffinity analog 8-azido-[\(\alpha^32\text{P}\)]ATP and, under equilibrium conditions, [\(\alpha^32\text{P}\)]ATP. We used [\(\alpha^32\text{P}\)]ATP, a transport substrate of Pgp, which is also a photoaffinity reagent, has also been used in these studies in a manner that recognizes the concerns associated with photoaffinity probes. [\(\alpha^32\text{P}\)]IAAP is used to understand the "coupling" between the ATP sites and the drug substrate site. [\(\alpha^32\text{P}\)]IAAP is used as a reporter molecule to monitor the state of the transport substrate site when the Pgp molecule has been trapped at different steps of the ATPase reaction. Thus, the actual titrations either thermal or kinetic were carried out under equilibrium conditions using the physiological substrate, ATP. These perturbations either do or do not result in conformational changes at the transport substrate site. In an enzymatic reaction, for example, such conformational changes may result in a product that can be measured very often by a chemical reaction. Similarly, in our assay, we allow the Pgp to be trapped in a stable, long lived ternary complex under equilibrium conditions and use [\(\alpha^32\text{P}\)]IAAP only to report on the conformational change at the transport substrate site. The temperature range (4–34 °C) used for the thermal titration had no effect on the photocross-linking reaction itself (see supplemental Fig. S1).

Estimating the Activation Energy for the Formation of Reaction Intermediates—The activation energy (E_{act}) for an enzymatic reaction is calculated from the slope of an Arrhenius plot, reciprocal of the absolute temperature versus log activity. Formation of the Pgp-ATP (in the mutant Pgp) or Pgp-ADP-Vi (in the wild-type Pgp) reaction intermediates is a slow process that occurs over several minutes (20, 22, 30). Moreover, we show that in the temperature range 10–34 °C, the trapping reaction is linear for at least 5 min (e.g. see Fig. S2 and Ref. 22). The trapping reactions depicted in this study were allowed to proceed for exactly 5 min, and the radioactivity of the trapped nucleotide in each sample as well as the protein at the end of the reaction was determined (28). Since all experiments were carried out using purified Pgp reconstituted into proteoliposomes, these measurements allowed us to express the extent of trapping as a rate measurement (viz. pmol nucleotide occluded/mg of Pgp/min). These values were used in generating the Arrhenius plots from which the E_{act} values in Table 1 were calculated. The procedure adopted to estimate the E_{act} value for nucleotide occlusion was essentially similar to that adopted by van der Does et al. (31).

RESULTS

The Mutant Pgp E556Q/E1201Q Exhibits Very Low Levels of ATP Hydrolysis but Occludes [\(\alpha^32\text{P}\)]ATP in the Transition State—Several groups have studied the role of the highly conserved glutamates within the Walker B region of the N- and the C-ATP sites of mouse (22) and human (20) Pgp. In human Pgp, the double mutant E556Q/E1201Q does not show steady state hydrolysis but can trap the nucleotide in the transition state (20), and the results were identical with equivalent mutations in mouse Pgp (22). Using crude membranes obtained from HeLa cells infected-transfected with mutant Pgp, we observed that 8-azido-[\(\alpha^32\text{P}\)]ATP was occluded in a manner similar to the Vi-induced trapping in wild-type Pgp. Moreover, we noted a diminished radioactive signal associated with the Pgp band when 8-azido[\(\gamma^32\text{P}\)]ATP was used in lieu of 8-azido[\(\alpha^32\text{P}\)]ATP and, under equilibrium conditions, [\(\alpha^32\text{P}\)]ATP.

attachment in the ATPase buffer (see above) with [\(\alpha^32\text{I}\)]IAAP (7 nm) for 5 min under subdued light. The samples were photocross-linked for 10 min at room temperature followed by electrophoresis and quantification as described previously (11). Modifications to this procedure in specific experiments are detailed in the legends to Figs. 4–6 and S1.

[\(\alpha^32\text{P}\)]ATP Hydrolysis—ATP hydrolysis was measured by determining the amount of [\(\alpha^32\text{P}\)]ADP released, as described previously (27). Purified Pgp reconstituted into liposomes (25–50 μg of protein/ml) was incubated with 200 μM [\(\alpha^32\text{P}\)]ATP for 20 min in the ATPase buffer (see above). Experiments were carried out in the absence and presence of Vi and 50 μM verapamil. The reaction was stopped by adding 100 μl of ice-cold buffer containing 2.5% SDS, and 1–2 μl of this sample was spotted onto a cellulose/polyethyleneimine membrane. TLC was carried out using 1 M HCOOH and 0.5 M LiCl as a solvent. The [\(\alpha^32\text{P}\)]ATP and [\(\alpha^32\text{P}\)]ADP were identified by exposing the dried TLC to an x-ray film. The [\(\alpha^32\text{P}\)]ATP and [\(\alpha^32\text{P}\)]ADP spots were quantified using the STORM 860 PhosphorImager system (Amersham Biosciences) and the software ImageQuaNT.

[\(\alpha^32\text{P}\)]ATP or [\(\alpha^32\text{P}\)]ADP Trapping—Purified wild type and E556Q/E1201Q mutant Pgps reconstituted into liposomes were incubated with 200 μM [\(\alpha^32\text{P}\)]ATP for 5 min in the ATPase buffer (see above). The reaction was stopped by adding 100 μl of ice-cold buffer containing 1 M NaCl and 10 mM ATP. The sample was loaded onto a G-50 column (4 ml, diameter 1 cm) previously washed with 50 mM Tris-HCl, pH 7.5, and 0.2 M NaCl (Buffer A). The proteoliposomes were eluted with Buffer A, 0.25-ml fractions were collected, and the elution of protein was monitored by measuring the OD at 280 nm. The fractions with the protein were pooled, diluted to 4 ml with cold Buffer A, and centrifuged at 150,000 × g for 20 min. The pellet was resuspended in 15 μl of 50 mM Tris-HCl, pH 7.5, containing 2.5% SDS. The amount of [\(\alpha^32\text{P}\)]nucleotide and protein, respectively, were estimated in each sample. We used the stock solution of [\(\alpha^32\text{P}\)]ATP to generate a standard curve, which was used to determine the concentration of occluded nucleotide. In each set of experiments, the purified and reconstituted wild-type Pgp was also incubated with [\(\alpha^32\text{P}\)]ATP at 4 °C in the absence of Vi. This is the negative control, where no trapping of nucleotide occurs (e.g. see Fig. 1B). The radioactivity obtained under these conditions after removal of the free [\(\alpha^32\text{P}\)]ATP (<0.5% of the signal obtained in the presence of Vi at 37 °C) was subtracted as background. The protein in each sample was estimated by the method of Schaffner and Weissmann (28), which was specifically developed to accurately measure the concentration membrane proteins in the presence of lipid or detergent. In some experiments (indicated in the figure legends), 1–2 μl of the sample was spotted onto a cellulose/polyethyleneimine membrane. TLC was carried out, and [\(\alpha^32\text{P}\)]ATP and [\(\alpha^32\text{P}\)]ADP were identified and quantified as described above.

Use of Photoaffinity Probes in Determining Kinetic and Thermodynamic Parameters—Our use of photoaffinity analogs has been criticized (29) on the grounds that these measurements are not carried out under equilibrium conditions. To allay these concerns, we have estimated the kinetic and thermodynamic parameters using both the photoaffinity analog 8-azido-[\(\alpha^32\text{P}\)]ATP and, under equilibrium conditions, [\(\alpha^32\text{P}\)]ATP.
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\[ ^{32}\text{P}]\text{ATP} \] and concluded that a single hydrolysis event may have occurred prior to the nucleotide being trapped (20). Senior and co-workers (22), on the other hand, allowed purified mouse Pgp expressed in Pichia pastoris to trap \([\alpha-^{32}\text{P}]\text{ATP}\), separated the nucleotides on a TLC, and found that in wild-type Pgp it was \([\alpha-^{32}\text{P}]\text{ADP}\) that was trapped, whereas in the mutant \(\sim 80\% \ [\alpha-^{32}\text{P}]\text{ATP}\) and 20\% \([\alpha-^{32}\text{P}]\text{ADP}\) were trapped. Since the latter is the more direct method, we overexpressed human Pgp (both wild-type and the E556Q/E1201Q mutant) in High Five insect cells, prepared crude membranes, purified the Pgp, and reconstituted into proteoliposomes as described under “Experimental Procedures.” The autoradiogram depicted in Fig. 1A shows that wild-type Pgp converts over 80\% of the \([\alpha-^{32}\text{P}]\text{ATP}\) to \([\alpha-^{32}\text{P}]\text{ADP}\) and that the ATP hydrolysis is sensitive to Vi. The mutant Pgp (E556Q/E1201Q), on the other hand, shows negligible ATP hydrolysis, and Vi has no effect on the reaction. Although an extremely sensitive method was used to estimate ATP hydrolysis, negligible \([\alpha-^{32}\text{P}]\text{ADP}\) formation could be detected in the presence of the mutant Pgp. These results are consistent with our findings with crude membrane preparations of human Pgp (20) and those with purified mouse Pgp (22). We then measured the occluded nucleotide in wild-type and mutant Pgps (Fig. 1B). Following incubation with \([\alpha-^{32}\text{P}]\text{ATP}\) at 37 °C, we stopped the reaction by adding excess cold ATP and removed the unbound nucleotides by column chromatography followed by centrifugation. The autoradiogram of the TLC (Fig. 1B) shows that wild-type Pgp does not occlude any nucleotide in the absence of Vi, whereas only \([\alpha-^{32}\text{P}]\text{ADP}\) is trapped in the presence of Vi. These results are all consistent with previously published results (20, 22). However, the results clearly show that the mutant Pgp traps nucleotide both in the absence and presence of Vi and that the nucleotide exists almost exclusively as the nucleoside triphosphate. These results using purified human Pgp agree with the finding of Senior and co-workers (22) using mouse Pgp. Our previous conjecture that it is the nucleoside diphosphate that is trapped (20) was based on indirect methods and the use of crude membrane preparations. A mutant of Pgp with drastically reduced ATPase activity that can occlude the nucleoside triphosphate suggests that it may be possible to arrest the catalytic cycle of ATP hydrolysis in a prehydrolysis reaction intermediate.

The E556Q/E1201Q Mutant and Wild-type Pgps Show Comparable Affinities for Nucleotides—As shown in Fig. 1, the mutant Pgp occludes \([\alpha-^{32}\text{P}]\text{ATP}\) such that it cannot be exchanged by a 50-fold excess of cold ATP. The wild-type Pgp also occludes the nucleotide, but it is \([\alpha-^{32}\text{P}]\text{ADP}\) that is trapped and only in the presence of Vi. It is well established that wild-type Pgp has a relatively low affinity for ATP (25, 32, 33), but the conformational changes that accompany ATP hydroly-

![Figure 1](image1.png)

**FIGURE 1.** Hydrolysis of \([\alpha-^{32}\text{P}]\text{ATP}\) and occlusion of \([\alpha-^{32}\text{P}]\text{ATP}/\text{ADP}\) by wild-type and the E556Q/E1201Q mutant Pgps. A, ATP hydrolysis mediated by purified and reconstituted wild-type and mutant Pgps in the presence or absence of Vi was measured by determining the amount of \([\alpha-^{32}\text{P}]\text{ADP}\) generated after incubation with 0.2 mM \([\alpha-^{32}\text{P}]\text{ATP}\). The Pgp, either wild-type or mutant, was incubated with \([\alpha-^{32}\text{P}]\text{ATP}\) at 37 °C for 20 min, and the reaction was stopped by the addition of an equal volume of 5\% SDS. The reaction mixture (1 \mu l) was spotted on a TLC, and the nucleotides were separated using a mixture of 1 M HCOOH and 0.5 M LiCl as the solvent. B, amount and nature (\([\alpha-^{32}\text{P}]\text{ATP}\)/\([\alpha-^{32}\text{P}]\text{ADP}\)) of the nucleotide occluded in the wild-type and mutant Pgps in the presence or absence of Vi. The reconstituted Pgps were incubated with \([\alpha-^{32}\text{P}]\text{ATP}\) as described in A. The reaction was stopped by adding 0.1 ml of ice-cold ATPase buffer containing 10 mM ATP and 1 M NaCl. The nucleotides and proteoliposomes were separated by gel chromatography followed by centrifugation. The autoradiograms were dried and exposed to an x-ray film. The positions of the \([\alpha-^{32}\text{P}]\text{ATP}\) and \([\alpha-^{32}\text{P}]\text{ADP}\) spots and the experimental conditions are depicted on the autoradiograms. These autoradiograms are typical of three independent experiments.

![Figure 2](image2.png)

**FIGURE 2.** Determination of the affinities of nucleotides for wild-type and the E556Q/E1201Q mutant Pgps. A, the apparent \(K_{d}\) (8-azido-ATP) was estimated by determining the photolabeling of reconstituted purified Pgps in the presence of increasing concentrations of 8-azido-(\[\alpha-^{32}\text{P}]\text{ATP}) at 4 °C, as described under “Experimental Procedures.” The radioactivity incorporated in the Pgp bands was estimated as arbitrary units using the STORM 860 PhosphorImager system and the software ImageQuaNT. The apparent \(K_{c}\) values were estimated using the curve-fitting software GraphPad PRISM. B, the apparent \(K_{c}\) (8-azido-ATP)/ADP for occlusion of nucleotide was determined as described in A above with the following differences. The Pgps were incubated with increasing concentrations of 8-azido-(\[\alpha-^{32}\text{P}]\text{ATP}) at 34 °C instead of 4 °C for 5 min. Moreover, whereas the wild-type Pgp was incubated with 8-azido-(\[\alpha-^{32}\text{P}]\text{ATP}) plus 250 \mu M Vi, the mutant was incubated with 8-azido-(\[\alpha-^{32}\text{P}]\text{ATP}) alone. The reaction was stopped by transferring tubes to ice and adding 10 mM ice-cold ATP prior to photocross-linking. C, the \(K_{c}\) for inhibition of 8-azido-(\[\alpha-^{32}\text{P}]\text{ADP}/\text{ATP}\) trapping by ATP was determined by incubating the Pgps with 30 \mu M 8-azido-(\[\alpha-^{32}\text{P}]\text{ATP}) and increasing concentrations of ATP at 34 °C for 5 min. Subsequent steps were carried out as described in B. In all experiments, 5 \mu g of Pgp protein (100 \mu g/ml) was used in each assay. All data points represent the mean \(\pm\) S.D. (represented by the error bars) of three independent experiments. The graphs depict incorporation of nucleotide in wild-type Pgp (●) and the E556Q/E1201Q mutant Pgp (○). Comparable results were also obtained using crude membranes prepared from High Five insect cells overexpressing wild-type and mutant Pgps (data not shown).

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sis allow the nucleotide diphosphate to be occluded in the presence of Vi (3, 18). Vi mimics the pentacovalent phosphorus, and the long lived, noncovalent ternary complex Pgp-MgADP-Vi is analogous to the catalytic transition state, Pgp-MgADP-Pi (18, 34, 35). The [α-32P]ATP-trapped state of the mutant Pgp could arise due to increased affinity for nucleotides or could be a reaction intermediate that is a consequence of conformational changes in the protein. To address this question, we first estimated the affinities of wild-type and mutant Pgp for nucleotides using different techniques. We compared the apparent $K_d$ (8-azido-ATP) for the binding of 8-azido-[α-32P]ATP at 4 °C. Fig. 2A shows that both wild-type and the E556Q/E1201Q mutant Pgps have comparable affinities (11.07 ± 0.4 μM and 10.7 ± 0.28 μM, respectively). However, the caveat is that 8-azido-ATP is an analog of ATP, and there is an additional photocross-linking step involved in this method. Estimating the $K_m$ would be a direct way to compare the affinities of the physiological nucleotide ATP, but this is impossible, since the mutant Pgp exhibits drastically reduced ATP hydrolysis. We thus monitored the IC50 (ATP) for the inhibition of 8-azido-[α-32P]ADP/ATP trapping. We first established that both wild-type and mutant Pgp had comparable apparent $K_m$ values (13.9 ± 2.9 and 18.64 ± 1.8 μM) for trapping (Fig. 2B), either in the presence of Vi (wild type) or absence of Vi (mutant). We then allowed nucleotide trapping to occur in the presence of increasing concentrations of ATP and monitored the decrease in the 32P signal in the Pgp band. Fig. 2C shows that ATP inhibits 8-azido-[α-32P]ADP trapping in wild-type Pgp with an IC50 = 0.29 ± 0.07 mM, and the IC50 (ATP) for inhibition of 8-azido-[α-32P]ATP trapping by mutant Pgp is 0.14 ± 0.06 mM. Moreover, these values are only slightly lower than the values reported for the $K_m$ (ATP) during ATP hydrolysis (9, 25, 32, 33). These results show that there is not a drastic increase in the affinity of nucleotides for the mutant Pgp that would explain the occlusion of ATP in the absence of Vi or BeF2.

The Energetics of Nucleotide Trapping in the E556Q/E1201Q Mutant and Wild-type Pgps—The data in Fig. 2 suggest that the Glu → Gln mutant Pgp attains the ATP-trapped state via a conformational change and is not a consequence of inherent greater affinity for nucleotide(s). This raises the question of whether the ATP-trapped state in the mutant Pgp is a reaction intermediate in the catalytic cycle of wild-type Pgp that has previously not been recognized. Thus, it is plausible that the extensively studied Pgp-MgADP-Vi reaction intermediate may have attained its particular conformation prior to ATP hydrolysis but could not be experimentally trapped. Whereas it is impossible to trap the Pgp-MgATP transition state of the wild-type protein in the absence of Vi or other transition state analogs of P+, the mutant Pgp, E556Q/E1201Q, by preventing cleavage of the γ-phosphate, permits the experimental characterization of the Pgp (E556Q/E1201Q)-MgATP reaction intermediate. Fig. 3A is a thermal titration of 8-azido-[α-32P]ADP/ATP trapping into wild-type and mutant Pgp in the absence and presence of Vi. There is minimal trapping of 8-azido-[α-32P]ADP into wild-type Pgp in the absence of Vi, and there is no influence of temperature, which is consistent with previous studies (19). In the presence of Vi, on the other hand, there is a strong temperature-dependent occlusion of 8-azido-[α-32P]ADP with an activation energy that is comparable with values reported previously (Table 1) (19). The mutant Pgp traps 8-azido-[α-32P]ATP both in the absence and presence of Vi, and this trapping is strongly dependent on temperature. Most important is the observation that the curves for the temperature-dependent increase in 8-azido-[α-32P]ADP/ATP trapping by
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**TABLE 1**

| Reaction | Nucleotide trappinga | Inhibition of IAAP bindingb |
|----------|-----------------------|-----------------------------|
| Azido-ATP/ADP | ATP/ADP | kJ/mol | |
| Vi-induced nucleotide trapping in wild-type Pgp | 65.3 ± 9.7 | 43 ± 7.9 | 49.3 ± 8.8 |
| BeF2-induced nucleotide trapping in wild-type Pgp | 62.4 ± 10.3 | 33.4 ± 5.9 | 44.1 ± 6.1 |
| Vi- or BeF2-independent nucleotide trapping in mutant Pgp (E556Q/E1201Q)c | 63.4 ± 8.3 | 37 ± 4.3 | 44.1 ± 5.7 |

a Nucleotide trapping was initiated by using either 8-azido-[α-32P]ATP or [α-32P]ATP.

b Binding of [125I]IAAP was measured after nucleotide trapping was initiated by using 1 mM ATP. The incubation with wild-type Pgp was carried out in the presence of Vi and in the absence of Vi for the E556Q/E1201Q mutant Pgp.

c The trapped moiety is either [α-32P]ADP or 8-azido-[α-32P]ADP.

d The trapped moiety is either [α-32P]ATP or [α-32P]ADP (see Fig. 1B).

Activation energy (Ea) values for the formation of different reaction intermediates during Pgp-mediated ATP hydrolysis

Activation energy (Ea) = −(slope) 2.3R. Slope was obtained from an Arrhenius plot, which is the reciprocal of the absolute temperature versus log activity. Formation of the Pgp-ATP (in the E556Q/E1201Q mutant Pgp) or Pgp-ADP-Vi (in the wild-type Pgp) reaction intermediates is a slow process that occurs over several minutes (see supplemental Fig. S2). This reaction is linear for at least 5 min in the temperature range 10–30 °C; thus, trapping reactions were allowed to proceed for exactly 5 min, and the activity was expressed as pmoL of nucleotide occluded/mg of Pgp/min (where [α-32P]ATP was used as the nucleotide). When 8-azido-[α-32P]ATP was used as the nucleotide, we estimated radioactivity in the Pgp band using a PhosphorImager, and activity was expressed as 8-azido-[α-32P]ATP/ADP incorporated (in arbitrary units)/mg of Pgp/min. See "Experimental Procedures" and legends to Figs. 3, 4, and 6 for details. The Arrhenius plots were generated using mean values of three independent experiments, and the linear regression (r) values were in the range of 0.88–0.99. Values represent mean ± S.D. (n = 3).

wild-type and mutant Pgp (Fig. 3A) as well as the activation energy values (Table 1) are indistinguishable. Although trapping of 8-azido-ATP has been extensively used to understand the transport cycle of ABC proteins (see Refs. 3 and 4 for reviews), the additional photocross-linking step and the fact that the use of photoaffinity reagents does not represent equilibrium conditions are matters of concern (e.g. see Ref. 29). To address these concerns, we monitored the effect of temperature on nucleotide trapping in wild-type and mutant Pgps using [α-32P]ATP. The results, depicted in Fig. 3B, are comparable with our findings with 8-azido-[α-32P]ATP (Fig. 3A). These data show that temperature enhances the nucleotide trapping in both wild-type and mutant Pgps. Moreover, these data support the proposition that the mutant Pgp occludes ATP as a reaction intermediate, Pgp(E556Q/E1201Q)-MgATP, which may be equivalent to the Pgp-MgATP (or the E-S) state in the catalytic cycle of Pgp.

The Reaction Intermediate in both Wild-type and Mutant Pgps Shows Reduced Binding of Transport Substrate [125I]IAAP—Earlier work from our laboratory (11) demonstrated that the Vi-trapped conformation of wild-type Pgp binds the photoaffinity transport substrate analog, [125I]IAAP with a >30-fold reduced affinity. These studies suggested that the reaction intermediate, Pgp-MgADP-Vi is arrested following a conformational change in the transport substrate binding from the high affinity, "on" site to a low affinity "off" site (11, 12). If the hydrolysis of ATP is the driving force for the conformational changes that affect the transport substrate binding site, the decrease in [125I]IAAP binding would have the same energetic requirements as the trapping of nucleotide. Moreover, it has been postulated that mutants of the catalytic carboxylate occlude ATP and that this nucleotide-driven dimerization provides the power stroke for an ABC transporter (5). However, these studies have not experimentally probed the transport substrate site while the ATP is occluded at the NBD(s). Thus, to determine the step(s) in the ATPase reaction associated with the switch at the transport substrate site, we monitored the binding of the transport substrate, [125I]IAAP, under conditions that permit formation of the reaction intermediates equivalent to the prehydrolysis E-S transition state in the mutant Pgp and the posthydrolysis E-P transition state with wild-type Pgp in the presence of Vi (see Figs. 1 and 3). Fig. 4A shows thermal titrations where wild-type Pgp was incubated with 1 mM ATP plus 0.25 mM Vi and the mutant Pgp was incubated with ATP alone for 5 min at the indicated temperatures. The trapping reaction was stopped by transferring to ice, and the transport substrate site was probed using [125I]IAAP. It must be emphasized that the conformational change at the transport substrate site was brought about by trapping Pgp at different steps in the ATPase reaction. This was carried out under equilibrium conditions using the physiological nucleotide ATP. Since the photocross-linking of [125I]IAAP was carried out at a fixed temperature (4 °C), the decrease in cross-linking cannot be attributed to an effect of temperature on the efficiency of cross-linking. We have demonstrated previously that the decreased binding of [125I]IAAP to Pgp in the Vi-induced transition state represents a 30-fold decrease in the affinity of [125I]IAAP for Pgp (12), and similar results were obtained using 3H-vinblastine (8) in the absence of cross-linking. The results depicted in Fig. 4A show that formation of the E-S and E-P reaction intermediates of the ATPase reaction is strongly temperature-dependent, and formation of either reaction intermediate results in a decrease in the affinity of the transport substrate for Pgp. Additionally, when the extent of trapping of nucleotide was plotted against the decrease in [125I]IAAP binding (Fig. 4B), an excellent correlation was obtained (r = 0.98 and 0.88 for the wild-type and mutant Pgps, respectively). Thus, the two events, generating the Pgp-MgATP or Pgp-MgADP reaction intermediate and effecting the conformational changes that result in decreased binding of the transport substrate are tightly coupled. We would, however, like to caution that due to the low concentration of [125I]IAAP used and the cross-linking efficiency, this assay cannot be used to estimate the stoichiometry of nucleotide trapping and [125I]IAAP binding.

Kinetics of Nucleotide Occlusion and Inhibition of [125I]IAAP Binding in the E556Q/E1201Q Mutant Pgp—We have demonstrated above that the formation of the E-S and E-P states of the ATPase reaction and the concomitant decrease in binding of...
The transport substrate $[^{125}\text{I}]$IAAP have similar energetics. We also monitored the occlusion of $[^{32}\text{P}]}$ATP/ADP in the mutant and wild-type Pgps as a function of ATP concentration (Fig. 5A). Since this assay was performed using purified Pgps and $[^{32}\text{P}]}$ATP, the molar ratio of protein/occluded nucleotide could be determined at each concentration of $[^{32}\text{P}]}$ATP. This experiment provides two important pieces of information: (i) the wild-type and mutant Pgps have comparable kinetics of nucleotide trapping (apparent $K_m$ values are 0.2 and 0.22 mM, respectively); and (ii) more noteworthy is the fact that when the data are fit to the Michaelis-Menten equation, the $B_{\text{max}}$ is $\sim 1$. Since the $y$ axis in this plot represents the ratio of pmol of nucleotide occluded/pmol of Pgp, this result suggests that the maximal stoichiometry (nucleotide/protein) is $\sim 1$ in both the $E$-$S$ and $E$-$P$ stages of the ATPase reaction under these experimental conditions. This value could be sensitive to the orientation and functional status of Pgp molecules in the proteoliposomal bilayer. We have previously demonstrated that $\geq 95\%$ (range 95–97\%) of Pgp molecules are reconstituted with an inside-out orientation (25, 26). For purification and reconstitution of the wild type and E556Q/E1201Q double mutant, we used protocol that allows $>90\%$ recovery of verapamil-stimulated ATPase activity (23, 25, 26). The specific activity of verapamil-stimulated ATPase activity in proteoliposomes prepared with various batches of pure wild-type protein ranged between 1100 and 1300 nmol of P/min/mg of protein as previously described (23). In addition, when we fit the curves taking into account these values, we found that the maximal stoichiometry...
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of mol of trapped nucleotide/pmol of Pgp is in the range of 0.94–1.04 for wild type Pgp and 1.15–1.23 for the mutant Pgp.

We also determined the kinetics of the decrease in binding of transport substrate \([^{125}\text{I}]\text{IAAP}\) (Fig. 5B) as a function of nucleotide occlusion. Here again we emphasize that the conformational change was brought about by using the physiological substrate, ATP at 34 °C, and the reaction was stopped by transferring the tubes to ice, and the \([^{125}\text{I}]\text{IAAP}\) was then used to monitor the transport substrate site at 4 °C. There was a clear decrease in the binding of \([^{125}\text{I}]\text{IAAP}\) as a function of ATP concentration. This experiment reinforces the idea that occlusion of nucleotide at the NBDs is coupled to conformational changes at the transport substrate site and that occlusion of 1 mol of ATP/mol of Pgp is sufficient for maximal inhibition of IAAP incorporation into the transporter.

The Beryllium Fluoride-trapped Conformation in Wild-type Pgp May Represent the E-S Reaction Intermediate—The principal reason that the Vi-induced complex in phosphatases is regarded as the posthydrolysis reaction intermediate is the trigonal-bipyramidal geometry of this complex in several x-ray crystallographic studies (17). Although the BeF\(_2\)-trapped complex of ATPases appears superficially like the Vi-trapped conformation in that both trap the nucleoside diphosphate, the geometry of the BeF\(_2\)-trapped complexes classifies it as a prehydrolysis reaction intermediate. Thus, for example, it was recently possible to obtain for the first time the structure of the enzyme-substrate complex of the bovine F\(_1\)-ATPase (36) that had long eluded structural biologists. This E-S complex was obtained using BeF\(_2\) and ADP. The biochemical diagnostic that distinguishes the prehydrolysis (BeF\(_2\)-trapped) and posthydrolysis (Vi-trapped) reaction intermediates is the fact that the formation of the former can be inhibited by PP\(_1\) (37). Comparing the effects of Vi- and BeF\(_2\)-induced trapping on \([^{125}\text{I}]\text{IAAP}\) binding in wild-type Pgp thus offers an alternative means of characterizing the E-S and E-P states of Pgp. Fig. 6A clearly shows that ATP inhibits \([^{125}\text{I}]\text{IAAP}\) binding to wild-type Pgp in the presence of either BeF\(_2\) or Vi. However, whereas PP\(_1\) can reverse the inhibition of \([^{125}\text{I}]\text{IAAP}\) binding by BeF\(_2\)-induced trapping, it has no effect on Vi-induced trapping (see lanes 7 and 3 in Fig. 6A). These results support the proposition that Pgp in the E-S conformation shows reduced binding of the transport substrate analog \([^{125}\text{I}]\text{IAAP}\). Moreover, thermal titrations of BeF\(_2\)-induced \([\alpha-3\text{P}]\text{ADP}\) trapping and inhibition of \([^{125}\text{I}]\text{IAAP}\) binding in the BeF\(_2\)-trapped state (Fig. 6B) are comparable with those for Vi-induced trapping (Figs. 3 and 4). Additionally, Table 1 shows that the activation energy for BeF\(_2\)-induced trapping (33.4 kJ/mol) is comparable with that obtained for Vi-independent trapping in the E556Q/E1201Q mutant Pgp (37 kJ/mol). The characterization of the BeF\(_2\)-induced \([\alpha-3\text{P}]\text{ADP}\)-trapped state suggests an alternative strategy to capture the E-S reaction intermediate and that it exhibits conformational changes comparable with Vi-independent trapping in the Walker B Glu → Gln mutant Pgp.

DISCUSSION

Understanding how the catalytic cycle of ATP hydrolysis is coupled to conformational changes at the transport substrate binding sites is one of the major goals of studies with ABC transport proteins. The catalytic mechanism of Pgp has been intensely studied (3–5), and although the fact that ATP hydrolysis is critical for transport in ABC transport proteins is well established (for reviews, see Refs. 3, 4, 7, 13, 14, and 38), precise steps in the ATPase reaction that bring about conformational changes in the transport substrate binding site are still not well understood (6, 8, 10, 11, 39). Capturing and studying the transient molecular forms that lie on the pathway between the initial and final states of enzymes has proved invaluable in understanding many catalytic mechanisms. In this study, we (i) characterize and define reaction intermediates of Pgp-mediated ATP hydrolysis based on their activation energies and the nature of the occluded nucleotide and (ii) exploit these reaction intermediates to identify the steps during the ATPase reaction where the high affinity → low affinity switch at the transport substrate site occurs.
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FIGURE 7. Progression of the Pgp-mediated ATPase reaction and accompanying conformational changes. A simplified scheme for the ATPase reaction of Pgp (E + S → S → P → E + Pi) is shown at the top. The E556Q/E1201Q mutant Pgp can trap [α-32P]ATP in the absence of Vi, which is an analog of P, (Fig. 2). Reaction intermediates have affinities 1010 to 1015 times higher than the corresponding Michaelis-Menten constants (Km) for human Pgp (Table 1), a methodology similar in principle to ours was used to estimate the Eact for the formation of the ATP-driven E599Q-NBD dimer of Mdr1 (an intracellular peptide transporter from Saccharomyces cerevisiae), and a value of 68 kJ/mol was obtained (31). Although the Eact values reported in Table 1 do not directly provide mechanistic information vis-à-vis the transport cycle, the values are sufficiently high to conclude that the nucleotide-occluded state results from relatively large conformational changes (41, 42). Our results also suggest that the nucleoside-trapped states of the wild type and mutant Pgps exhibit characteristics of reaction intermediates. Additionally, the trapped species in the mutant Pgp is the nucleoside triphosphate (Fig. 1B) (22) and in all likelihood represents a reaction intermediate equivalent to the E-S state during Pgp-mediated ATP hydrolysis. Similarly, the Pgp-ADP-Vi complex also shows properties diagnostic of reaction intermediates discussed above; viz. occluded and nonexchangeable nucleotide, a slow onset (supplemental Fig. S2) (12, 19), and a high activation energy (Table 1) (19). In addition, there are x-ray crystallographic structures of 39 Vienzyme complexes in the Protein Data Base, of which 21 have been clearly identified as representing reaction intermediates (17). Recently, for example Chen et al. have determined the crystal structure of the mitochondrial F1-ATPase in a vanadate-induced transition-like state (44). Moreover, the nucleotide moiety trapped in Vi-trapped structures is always the nucleoside diphosphate (Fig. 1A) (3, 4), suggesting that it would be reasonable to designate the Vi-trapped reaction intermediate of Pgp as equivalent to the E-Pstate of the ATPase reaction.

Reaction intermediates that represent the transition states during an enzymatic reaction can be captured in two ways. The first is to modify the enzyme, often by mutations in the active site, and the second is to use transition state inhibitors (16). The E556Q/E1201Q mutant Pgp can trap [α-32P]ATP in the absence of Vi (Fig. 2) (20, 22), and we demonstrate in this study that this is not due to altered affinities for nucleotides (Fig. 2). Reaction intermediates have affinities 1010 to 1015 times higher than the corresponding Michaelis-Menten complexes (16), reflected in the Eact values for both nucleotide trapping and decrease in [125I]IAAP binding following trapping (Fig. 1A) (3, 4). The Eact values for both nucleotide trapping and decrease in [125I]IAAP binding following trapping was in the range of 37–50 kJ/IAAP (Table 1). A methodology similar in principle to ours was used to estimate the Eact for the formation of the ATP-driven E599Q-NBD dimer of Mdr1 (an intracellular peptide transporter from Saccharomyces cerevisiae), and a value of 68 kJ/mol was obtained (31). Although the Eact values reported in Table 1 do not directly provide mechanistic information vis-à-vis the transport cycle, the values are sufficiently high to conclude that the nucleotide-occluded state results from relatively large conformational changes (41, 42). Our results also suggest that the nucleoside-trapped states of the wild type and mutant Pgps exhibit characteristics of reaction intermediates. Additionally, the trapped species in the mutant Pgp is the nucleoside triphosphate (Fig. 1B) (22) and in all likelihood represents a reaction intermediate equivalent to the E-S state during Pgp-mediated ATP hydrolysis.
findings suggest that the reaction intermediates exhibit structural features as well as biochemical properties of the transition states. Finally, this methodology has provided evidence that conformational changes in the nucleotide domain observed in the reaction intermediates of the ATPase reaction are transmitted to the motor domain (46–48, 51). In our studies, we have monitored the conformational changes at the transport substrate site of Pgp arrested at different steps of the ATPase reaction (Fig. 7). The nucleotide binding per se (i.e. binding of ATP or AMPPNP at 4 °C) (11) does not affect the transport substrate site (Step I), and there is no energy barrier associated with this step (Fig. 3, Table 1) (19). The formation of the E*S complex (Step II) is accompanied by a high → low affinity switch in the transport substrate site (Figs. 4–6). This is logical, because transition state theory holds that attaining the E*S transition state requires energy for alignment of reacting groups, formation of transient unstable charges, bond rearrangements, and other conformational changes (54). We are aware of the fact that some form of nucleotide-induced dimerization observed in bacterial ABCs (with isolated NBDs) has led to the postulate that ATP binding provides the power stroke for the pump and that hydrolysis drives the thermodynamic destabilization of the dimer and enables the pump to turn over or “reset” (5, 56). However, these studies have not measured the consequences of nucleotide occlusion on the transport substrate binding site. Here we show that (i) nucleotide occlusion exhibits Michaelis-Menten kinetics and is temperature-dependent (suggesting conformational changes) and (ii) the inhibition of binding of the transport substrate [125I]IAAP is strongly coupled to occlusion of the nucleotide. The nucleotide-driven dimerization observed in bacterial ABCs (with isolated NBDs) has led to the postulate that ATP binding provides the power stroke for the pump and that hydrolysis drives the thermodynamic destabilization of the dimer and enables the pump to turn over or “reset” (5). If looked at in the framework provided by Fig. 7, the ATP switch and sandwich dimer model would require that the “high → low affinity switch” occur at Step II and “resetting” occur at Step IV (i.e. both events occur in the course of a single ATP hydrolysis event). Although the “high affinity → low affinity switch” at the transport substrate site occurs during formation of the E*S state, the E*P state of Pgp continues to show low affinity for transport substrates (Fig. 4). What is more important, most of the data in the literature suggest that the Vi-induced ADP-trapped state of Pgp exists in a conformation that shows reduced transport substrate affinity (8, 10, 11, 39). Qu et al. (29) have, however, suggested (using soluble Pgp in detergent solution) that the release of drug from the transporter during the catalytic cycle precedes formation of the transition state (where they refer to the Vi-trapped state of wild-type Pgp). We suggest that the apparent contradiction in the literature vis-à-vis the source of the energy for the power stroke could be resolved if the binding event that affects the switch is regarded as the E*S state, an early event during, but importantly not independent of, ATP hydrolysis. This concept is supported by recent work to understand how enzymes surmount the activation energy barrier that explicitly recognizes that the “microhistory” of an enzyme-catalyzed process begins with the E*S complex (59).

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