Functionally Similar Vanadate-induced 8-Azidoadenosine 5’-[α-32P]Diphosphate-trapped Transition State Intermediates of Human P-glycoprotein Are Generated in the Absence and Presence of ATP Hydrolysis

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P-glycoprotein (Pgp) is an ATP-dependent drug efflux pump whose overexpression confers multidrug resistance to cancer cells. Pgp exhibits a robust drug substrate-stimulable ATPase activity, and vanadate (Vi) blocks this activity effectively by trapping Pgp nucleotide in a non-covalent stable transition state conformation. In this study we compare Vi-induced [α-32P]8-azido-ADP trapping into Pgp in the presence of [α-32P]8-azido-ATP (with ATP hydrolysis) or [α-32P]8-azido-ADP (without ATP hydrolysis). Vi mimics P, to trap the nucleotide tenaciously in the Pgp-[α-32P]8-azido-ADP-Vi conformation in either condition. Thus, by using [α-32P]8-azido-ADP we show that the Vi-induced transition state of Pgp can be generated even in the absence of ATP hydrolysis. Furthermore, half-maximal trapping of nucleotide into Pgp in the presence of Vi occurs at similar concentrations of [α-32P]8-azido-ATP and [α-32P]8-azido-ADP. The trapped [α-32P]8-azido-azido-ADP is almost equally distributed between the N- and the C-terminal ATP sites of Pgp in both conditions. Additionally, point mutations in the Walker B domain of either the N- (D555N) or C (D1200N)-terminal ATP sites that arrest ATP hydrolysis and Vi-induced trapping also show abrogation of [α-32P]8-azido-ADP trapping into Pgp in the absence of ATP hydrolysis. These data suggest that both ATP sites are dependent on each other for function and that each site exhibits similar affinity for 8-azido-ATP (ATP) or 8-azido-ADP (ADP). Similarly, Pgp in the transition state conformation generated with either ADP or ATP exhibits drastically reduced affinity for the binding of analogues of drug substrate ([125I]iodoarylazidoprazosin) as well as nucleotide (2(3′)-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate). Analyses of Arrhenius plots show that trapping of Pgp with [α-32P]8-azido-ADP (in the absence of hydrolysis) displays an ~2.5-fold higher energy of activation (152 kJ/mol) compared with that observed when the transition state intermediate is generated through hydrolysis of [α-32P]8-azido-ATP (62 kJ/mol). In aggregate, these results demonstrate that the Pgp-[α-32P]8-azido-ADP (or ADP)-Vi transition state complexes generated even in the absence of or accompanying [α-32P]8-azido-ATP hydrolysis are functionally indistinguishable.

Cancer cells resistant to chemically diverse drugs with multiple mechanisms of action are defined as exhibiting the multiple drug resistance (MDR) phenotype. The best defined form of MDR in human cells is due to the overexpression of P-glycoprotein (Pgp). This 170-kDa plasma membrane protein is a member of the ATP-binding cassette (ABC) superfamily of transport proteins, and can extrude a range of hydrophobic anticancer drugs from cells against a concentration gradient and thus render cells resistant to cytotoxic chemotherapeutic agents. Analysis of hydrophathy plots suggests that Pgp consists of two homologous halves each containing six transmembrane helices and one nucleotide-binding or ATP site in each half (1, 2).

The extrusion of cytotoxic agents is powered by ATP hydrolysis, and the ATPase activity of Pgp has been studied in considerable detail. [α-32P]8-azido-ATP, a radiolabeled, photoaffinity analogue of ATP, has proved to be a valuable reagent in understanding interactions between nucleotide and Pgp (3–7). The use of [α-32P]8-azido-ATP along with orthovanadate (Vi) has permitted experimental strategies to elucidate the catalytic cycle (3, 5, 7–12). Vi inhibits Pgp ATPase activity by trapping nucleotide in the catalytic site to generate the transition state conformation, Pgp-ADP-Vi. It has been established that it is always a nucleoside diphosphate that is the trapped species (3, 13). Thus if ATP (or 8-azido-ATP) is used to initiate the reaction, at least one turnover of ATP hydrolysis, converting ATP to ADP, is essential for trapping to occur. This has allowed Vi-trapping experiments to be used to construct a catalytic scheme for ATP hydrolysis by Pgp.

Our recent work (10–12) has considerably expanded the original model for the catalytic scheme of Pgp proposed by Senior and co-workers (14). Our data suggest that ATP hydrolysis at one of the two ATP sites results in a dramatic conformational change where the affinities of both the substrate and the nucleotide for Pgp are drastically reduced. The fact that ATP binding to the second site is arrested while the first one is in a catalytic conformation appears to be the basis for alternate catalysis in Pgp (12). Moreover, for Pgp to regain the conformation that binds substrate with high affinity, the hydrolysis of an additional molecule of nucleotide is obligatory. Finally, we showed that release of ADP from the Pgp-ADP-Pi transition state is the rate-limiting step in the catalytic cycle of ATP hydrolysis (11).

Much of the work described above has benefited from the...
availability of [\alpha^{32}P]8-azido-ATP allowing direct visualization and quantification of the nucleotide interaction with Pgp. However, as only the photoaffinity analogue of nucleoside triphosphate was available as a radioisotope, this has precluded directly addressing many questions that require the [\alpha^{32}P]-labeled azido derivative of nucleoside diphosphate. In this study, we have characterized for the first time the binding and Vi-induced trapping of [\alpha^{32}P]8-azido-ADP to Pgp. We demonstrate that [\alpha^{32}P]8-azido-ADP binds specifically to Pgp with a $K_d$ comparable to that for [\alpha^{32}P]8-azido-ATP. The kinetic scheme for the Vi-induced inhibition of Pgp ATP hydrolysis proposed by Senior’s group (14, 15) suggests that incorporation of ADP into the Pgp-ADP-Vi ternary complex may occur either following hydrolysis of ATP to ADP or directly by the addition of ADP in the absence of hydrolysis.

We demonstrate in this study that it is possible to initiate Vi-induced trapping with either [\alpha^{32}P]8-azido-ADP or [\alpha^{32}P]8-azido-ATP, with similar kinetics, and that the trapped [\alpha^{32}P]8-azido-ADP distributed equally between the N- and the C-terminal halves of Pgp under both hydrolysis and non-hydrolysis conditions. Vi-induced trapping under both hydrolysis and non-hydrolysis conditions exhibited the same requirement for divalent cations. Previous work has demonstrated that a point mutation in the Walker B region (D555N and D1200N, respectively) of either the N- or C-terminal ATP sites arrests ATP hydrolysis and the Vi-induced trapping (6, 9). We find that these mutants also do not show Vi-induced [\alpha^{32}P]8-azido-ADP trapping into Pgp in the absence of hydrolysis, suggesting that the functional interaction of both ATP sites is necessary for the formation of the transition state intermediate. Our previous work demonstrated a direct interaction between the substrate and ATP sites by showing that the Vi-trapped intermediate exhibits drastically reduced affinity for the Pgp substrate analogue, [\textsuperscript{125}I]iodoarylazidoprazosin (IAAP) (5, 8, 10). We show here that the Vi-trapped intermediate generated with 8-azido-ADP, 8-azido-ATP, ADP, and ATP results in drastically reduced binding of IAAP to Pgp. Most interesting, however, analyses of the Arrhenius plots at steady state demonstrate that the activation energy for generating the Pgp-[\alpha^{32}P]8-azido-ADP-Vi transition state complex starting with [\alpha^{32}P]8-azido-ADP is $\approx 2.5$ times greater than if [\alpha^{32}P]8-azido-ATP were used, and it is 1.5 times greater than that required for the basal or verapamil-stimulated-stimulated hydrolysis of ATP or 8-azido-ATP. These data indicate that the Vi-trapped intermediate generated under hydrolysis or non-hydrolysis conditions do not show functional differences, and we suggest that the use of [\alpha^{32}P]8-azido-ADP should prove useful to compare the catalytic cycle of ATP hydrolysis by different ABC transporters.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**[\textsuperscript{125}I]IAAP, 2,200 Ci/mmol, was obtained from PerkinElmer Life Sciences. [\alpha^{32}P]8-Azido-ATP (15–20 Ci/mmol), [\alpha^{32}P]8-Azido-ADP (15–20 Ci/mmol), 8-azido-ATP, and 8-azido-ADP were purchased from Affinity Labeling Technologies, Inc. Pgp-specific monoclonal antibody C219 was a generous gift 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 Human MDR1 Gene—* High Five insect cells (Invitrogen, Carlsbad, CA) were infected with the recombinant baculovirus carrying the human MDR1 cDNA with a His$_6$ tag at the C-terminal end (BV-MDR1 (His)$_6$) as described previously (5). Crude membranes were prepared as described previously (5, 16).

**Photolabeling of Crude Membranes from HeLa Cells Expressing Mutant and Wild-type Pgp—** A 70–80% confluent monolayer of HeLa cells was infected with vTF7-3 and transfected with PTM1-MDR1 (wild type) or PTM1-MDR1 bearing the homologous mutation at positions 555 (D555N) and 1200 (D1200N) as described previously (9). The vaccinia virus expression vectors pTM1-MDR1 (wild type) and pTM1-MDR1(D555N) and pTM1-MDR1(D1200N) were provided by C. A. Hrycyna and M. M. Gottesman, Laboratory of Cell Biology, NCI, National Institutes of Health, Bethesda. Crude membranes were prepared from the HeLa cells as described previously (17).
have been established that the ATPase activity of Pgp follows simple Michaelis-Menten kinetics with a $K_m$ of ~0.3 to 1 mM depending on the source of Pgp (11, 23–25). The technical difficulties that this low affinity for nucleotide entails have been enumerated in detail by Senior and colleagues (24, 26), and the Vi-induced trapping of nucleotides into the ATP sites has proved to be an invaluable tool in elucidating the ATP hydrolysis cycle of Pgp. Vi is a potent inhibitor of the ATPase activity of Pgp, which acts by mimicking the pentacovalent phosphorus catalytic transition state, thereby trapping the nucleotide tenuously. This transition state conformation of Pgp has been characterized in earlier studies (3), by incubation of Pgp with $[\alpha^{32P}]$8-azido-ATP and Vi at 37 °C (i.e. allowing $[\alpha^{32P}]$8-azido-ATP to be hydrolyzed to $[\alpha^{32P}]$8-azido-ADP). Several lines of evidence have indicated that the trapped moiety is the nucleoside diphosphate (3, 4, 13), and Fig. 2A (after Senior et al. (14)) depicts the kinetic scheme for generating the Pgp-MgADP-Vi complex starting with Pgp, MgATP, and Vi. ATP binds to Pgp in the presence of Mg$^2+$ (step 1), and this is followed by hydrolysis (step 2) during which ATP is converted to ADP and P. Subsequently, P is released and ADP dissociates from Pgp. However, if Vi, an analogue of P, is present, it “traps” the ADP to form a stable, ternary, non-covalent complex, Pgp-MgADP-Vi (step 3). This complex eventually dissociates to Pgp + MgADP + Vi (step 4). As step 4 is a reversible process, in principle if MgADP were directly provided to Pgp, it would be stabilized by Vi into the Pgp-MgADP-Vi transition state complex (step 4b in boxed figure). Fig. 2B shows that if Pgp is incubated with 50 $\mu$M $[\alpha^{32P}]$8-azido-ADP and 250 $\mu$M Vi for 10 min at 37 °C, the $[\alpha^{32P}]$8-azido-ADP is occluded at the catalytic site (lane 3) and is not competed out by even 200-fold excess non-radioactive nucleotide (lane 4). This is in stark contrast to incubating Pgp in the presence of $[\alpha^{32P}]$8-azido-ADP but in the absence of Vi, where there is significantly less incorporation of $[\alpha^{32P}]$8-azido-ADP into Pgp suggesting that hydrolysis is not a prerequisite for Vi-induced trapping per se but is obligatory when $[\alpha^{32P}]$8-azido-ATP (or any other hydrolyzable nucleoside triphosphate) is used so as to generate the nucleoside diphosphate.

To characterize further Pgp in the transition state conformation generated by using $[\alpha^{32P}]$8-azido-ADP, we compared the distribution of the trapped $[\alpha^{32P}]$8-azido-ADP in the N- and the C-terminal ATP sites of Pgp. Fig. 2C demonstrates that consistent with previously published reports (9, 12, 27–29), the $[\alpha^{32P}]$8-azido-ADP distributes approximately equally between the N- and C-terminal halves of Pgp, and the distribution is similar regardless of whether the occluded $[\alpha^{32P}]$8-azido-ADP is generated through the hydrolysis of $[\alpha^{32P}]$8-azido-ATP or directly providing the nucleoside diphosphate itself.

**Effect of Divalent Cations on the Vi-induced Trapping of $[\alpha^{32P}]$8-Azido-ADP Under Hydrolysis and Non-hydrolysis Conditions**—The experiments described thus far have used magnesium as a metal cofactor with the nucleotide as it is well established that ATP binds to Pgp as an MgATP complex (30). However, several divalent cations such as manganese and...
balt are known to support ATPase activity (31), although with considerably reduced $V_{\text{max}}$ values vis-à-vis magnesium. The $V_{\text{max}}$ value for MnATPase is 43% that for MgATPase and only 10% for CoATPase (3). Studies have also demonstrated that replacing magnesium with other cations such as Mn$^{2+}$ and Co$^{2+}$ also support the Vi-induced trapping of [α-32P]8-azido-ADP (11, 27, 31). Compared with the extent of Vi-induced [α-32P]8-azido-ADP trapping in the presence of Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$, the amount of [α-32P]8-azido-ADP incorporated in the presence of Co$^{2+}$ was negligible (31). In Fig. 3 we compared the Vi-induced trapping of [α-32P]8-azido-ADP under hydrolysis conditions (i.e. initiating the reaction with [α-32P]8-azido-ATP and Vi) and non-hydrolysis conditions (i.e. initiating the reaction with [α-32P]8-azido-ADP and Vi). We find that Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$ permit Vi-induced trapping of [α-32P]8-azido-ADP under both hydrolysis and non-hydrolysis conditions. The extent of trapping under both conditions follows the pattern $\text{Mg}^{2+} > \text{Co}^{2+} > \text{Mn}^{2+}$. Ca$^{2+}$ does not support Vi-induced trapping of [α-32P]8-azido-ADP even in the absence of hydrolysis.

Comparing the Kinetics of Generating the Pgp[α-32P]8-Azido-ADP Transition State Intermediates Using Either [α-32P]8-Azido-ATP or [α-32P]8-Azido-ADP and Vi—We have shown above that it is possible to generate the Pgp[α-32P]8-azido-ADP-Vi transition state intermediate by directly incubating Pgp with [α-32P]8-azido-ADP and Vi. It would be important to determine the kinetics of Vi-induced [α-32P]8-azido-ADP

Fig. 2. [α-32P]8-Azido-ADP in the presence of Vi is trapped into a ternary complex with Pgp. A, catalytic scheme for the Vi-induced trapping of ADP into Pgp. The kinetic scheme presented here is based on published reports (11, 14, 27) Step 1, MgATP binds to Pgp in the presence of Mg$^{2+}$. Step 2, binding of MgATP is followed by hydrolysis; MgATP is converted to MgADP; P$_i$ is released, and MgADP dissociates from Pgp. Step 3, however, if Vi, an analogue of P$_i$, is present in the reaction, MgADP is trapped to form a stable, ternary, non-covalent complex, Pgp-MgADP-Vi. Step 4, eventually, MgADP and P$_i$ dissociate from Pgp. As Step 4 is a reversible process, in principle if MgADP were directly provided to Pgp then the Pgp-MgADP formed would be stabilized by Vi into the Pgp-MgADP-Vi transition state complex. This is depicted in the box at the bottom (Steps 4a and 4b) except that MgADP is replaced with Mg[α-32P]8-azido-ADP (see B). Note: although we depict the release of P$_i$ from the complex as concurrent with ATP hydrolysis (Step 2), it is very likely that there are several sub-states between the hydrolysis of MgATP and the release of P$_i$. More importantly, each of these could have subtle conformational differences, and agents such as Vi, aluminum fluoride, or beryllium fluoride could trap each of these in a ternary complex. B, [α-32P]8-azido-ADP incubated with Pgp in the presence of Vi results in [α-32P]8-azido-ADP being trapped into a ternary complex. Crude membranes (2 mg/ml) were incubated at 37 °C in the ATPase assay buffer containing 50 mM ATP and placing the sample on ice or by placing the sample on ice without adding ATP. The samples were then cross-linked by UV irradiation at 365 nm. An equal amount of protein (96 µg) was loaded in each lane. C, distribution of trapped [α-32P]8-azido-ADP in the N- and C-terminal ATP sites of Pgp. Crude membranes (2 mg/ml) were incubated in the ATPase assay buffer containing 50 mM [α-32P]8-azido-ADP and 250 mM Vi, and 12.5 mM ATP was added prior to UV cross-linking. The left and right panels are autoradiograms from the same gel; however, as the signals for lanes 3 and 4 were extremely high the gels were exposed to the x-ray film for different times. The left panel (lanes 1 and 2) were exposed to the x-ray film for 36 h at ~70 °C and the right panel (lanes 3 and 4) for 8 h. An equal amount of protein (96 µg) was loaded in each lane. C, distribution of trapped [α-32P]8-azido-ADP in the N- and C-terminal ATP sites of Pgp.
trapping under hydrolysis and non-hydrolysis conditions. The kinetics of trapping using \([\alpha-32P]8\text{-azido-ADP} + \text{Vi}\) or \([\alpha-32P]8\text{-azido-ADP} + \text{Vi}\) are depicted in Fig. 4, A and B. The incorporation of \([\alpha-32P]8\text{-azido-ADP}\) through the hydrolysis of increasing concentrations of \([\alpha-32P]8\text{-azido-ATP}\) or in the presence of increasing concentrations of \([\alpha-32P]8\text{-azido-ADP}\) itself exhibits Michaelis-Menten kinetics with a \(K_m\) of 20 ± 4.3 \(\mu\text{M}\) for \([\alpha-32P]8\text{-azido-ATP}\) and a \(K_v\) of 16 ± 1.6 \(\mu\text{M}\) for \([\alpha-32P]8\text{-azido-ADP}\), respectively. Thus, the affinity of \([\alpha-32P]8\text{-azido-ADP}\) for Pgp is similar under hydrolysis and non-hydrolysis conditions.

The Vi-induced ADP-trapped Conformation of Pgp Exhibits a Marked Decrease in Affinity for the Fluorescent Nucleotide TNP-ATP—We have demonstrated earlier that there is a reduced binding of nucleotide to Pgp in the transition state conformation (12). This can be demonstrated by first generating the Pgp-ADP-Vi transition state by incubating with ATP and Vi, washing off excess ATP and Vi, and then determining the extent of binding of TNP-ATP, a hydrolyzable, fluorescent analog of ATP (32), previously used to characterize the ATP site of Pgp (33, 34). Fig. 5 shows that when Pgp is pretreated with Vi and ATP or ADP at 37 °C, to generate the Pgp-ADP-Vi complex, there is a marked decrease in the binding of TNP-ATP to Pgp as evidenced by decreased levels of fluorescence. These results are consistent with our earlier finding that binding of \([\alpha-32P]8\text{-azido-ATP}\) to TNP-ATP to Pgp is drastically reduced when the transporter is trapped in the transition state conformation (12).

Mutations in the Walker B Domain of Either the N- or C-terminal ATP Sites of Pgp Arrest Vi-induced Trapping of \([\alpha-32P]8\text{-azido-ADP} Either in the Presence or Absence of ATP Hydrolysis—The experiments described above demonstrate that it is possible to generate the Pgp-[\alpha-32P]8-azido-ADP-Vi catalytic state intermediate by directly incubating Pgp with \([\alpha-32P]8\text{-azido-ADP}\) and Vi, and the kinetics are comparable regardless of whether \([\alpha-32P]8\text{-azido-ADP}\) or \([\alpha-32P]8\text{-azido-ATP}\) is used to initiate the Vi-induced trapping. It has also been demonstrated previously that if mutations are made in the conserved Walker B consensus motif in either the N- or C-terminal ATP sites of Pgp at positions Asp-555 and Asp-1200, respectively, which represent the putative magnesium-binding site, these mutants are unable to support ATP hydrolysis as well as the Vi-induced trapping of \([\alpha-32P]8\text{-azido-ADP}\) generated through the hydrolysis of \([\alpha-32P]8\text{-azido-ATP}\) (6, 9). If only ATP hydrolysis is affected in the mutants, using \([\alpha-32P]8\text{-azido-ADP}\) instead of \([\alpha-32P]8\text{-azido-ATP}\) should allow trapping of the nucleoside diphosphate. To test this hypothesis, we incubated crude membranes containing both wild-type and mutant Pgp with either \([\alpha-32P]8\text{-azido-ATP}\) or \([\alpha-32P]8\text{-azido-ADP}\) in the presence of Vi at 37 °C for 10 min. Excess ATP (200-fold) was added to all samples and then cross-linked by UV irradiation. The samples were cross-linked by UV irradiation at 365 nm and processed as described in A. The apparent affinity (\(K_d\)) of \([\alpha-32P]8\text{-azido-ADP}\) for Pgp in the presence of Vi was 16 ± 1.6 \(\mu\text{M}\).
we used both nucleoside tri- and diphosphates to trap Pgp in the transition state conformation. Crude membranes containing Pgp were incubated either with ATP, ADP, 8-azido-ATP, or 8-azido-ADP (1.25 mM) and 250 μM Vi at 37 °C in the dark. Aliquots were removed at intervals, incubated with IAAP, and cross-linked by UV irradiation. Following SDS-PAGE, the IAAP incorporated into the Pgp bands was quantified using a PhosphorImager. The results, depicted in Fig. 7, A–D, show that trapping of all the nucleotides tested inhibit IAAP binding in the presence of Vi. We have shown earlier that nucleotides in the absence of Vi or Vi in the absence of nucleotide do not affect IAAP binding (10). These results demonstrate the fact that in the presence of Vi and Mg2+, it is the nucleoside diphosphate that is trapped at the ATP site, and the resulting conformational changes are sufficient to effect changes in the substrate-binding site, resulting in the decreased affinity for substrate. Moreover, these data indicate that the transition state conformation of Pgp generated either in the presence or absence of hydrolysis of nucleoside is similar with respect to its effect on the substrate-binding site(s).

**The Inhibition of IAAP Labeling of Pgp during Vi-induced Trapping of 8-Azido-ATP and the Extent of Vi-induced Trapping of [α-32P]8-Azido-ADP Are Correlated—**To understand whether there is a cause-effect relationship between Vi-induced trapping of nucleoside diphosphate per se and the inhibition of substrate binding to Pgp, we performed the following two experiments in parallel. Crude membranes were incubated with 1.25 mM 8-azido-ADP and 250 μM Vi. Aliquots were removed at different time intervals, treated with IAAP for 5 min, and cross-linked by UV irradiation. In a parallel experiment the crude membranes were incubated with 50 μM [α-32P]8-azido-ADP and 250 μM Vi. Aliquots were removed at different time intervals, and 200-fold excess ATP was added followed by cross-linking with UV irradiation. The results of this experiment depicted in Fig. 8 show that the increased [α-32P]8-azido-ADP trapping over time is accompanied by decrease in IAAP binding. The inset shows that the two are inversely correlated (r = 0.92) suggesting that Vi-induced trapping of [α-32P]8-azido-ADP at the ATP site induces conformational changes that reduce the affinity of IAAP for Pgp.

**Determination of the Activation Energies for Vi-induced Trapping Using [α-32P]8-Azido-ATP and [α-32P]8-Azido-ADP—**The results thus far clearly show the following: (a) that nucleoside diphosphates in the presence of Mg2+ and Vi form a stable, ternary, non-covalent complex at the nucleotide-binding site of Pgp. The nucleoside diphosphate can be directly or as a nucleoside triphosphate that can be hydrolyzed in situ to a nucleoside diphosphate. (b) When the nucleoside diphosphate is trapped at the ATP site, the ternary complex manifests a profound conformational change at the substrate-binding site. This suggests that although the nucleotide- and substrate-binding sites are independent, long range interactions acting via conformational changes result in these two sites being functionally coupled. (c) The functional effect on the substrate-binding site is the same regardless of whether a nucleoside di- or triphosphate is used to initiate the trapping of Pgp into the Pgp-ADP-Vi transition state intermediate. This raises the question as to whether the two routes for generating the Pgp-ADP-Vi transition state are thermodynamically comparable. Fig. 9A depicts the effect of temperature on the Vi-induced trapping of [α-32P]8-azido-ADP. There is a low level of [α-32P]8-azido-ADP trapping at 23 °C, which is significantly increased at 37 °C, when Pgp is incubated with [α-32P]8-azido-ATP and Vi for 6 min. However, when [α-32P]8-azido-ADP and Vi are used to initiate trapping, there is no detectable trapping at 23 °C but a significantly increased level of incorporation of
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To understand this difference, we determined the activation energies for these two processes. Fig. 9B depicts Arrhenius plots for Vi-induced trapping using either \([\alpha^{32}P]8\text{-azido-ADP}\) or \([\alpha^{32}P]8\text{-azido-ATP}\) in the temperature range 22–39 °C. In this temperature range both the plots show no discontinuity, but the slopes are significantly different, and the steeper slope for the formation of the transition state intermediate with \([\alpha^{32}P]8\text{-azido-ADP}\) translates to a 2.5-fold higher activation energy (152 kJ/mol). A published report of the activation energy for ATP hydrolysis in crude membranes derived from the Chinese hamster ovary cell line, CHB30, shows a discontinuity at 21 °C (35). We could not perform our experiments at lower temperatures (<22 °C) as the trapping with \([\alpha^{32}P]8\text{-azido-ADP}\) and Vi even at 23 °C was undetectable (cf. Fig. 9A). Thus, if \([\alpha^{32}P]8\text{-azido-ADP}\) is trapped into Pgp by incubating \([\alpha^{32}P]8\text{-azido-ATP}\) and Vi, allowing the \([\alpha^{32}P]8\text{-azido-ATP}\) to be hydrolyzed to \([\alpha^{32}P]8\text{-azido-ADP}\), which is then trapped, the energy barrier is significantly lower than if \([\alpha^{32}P]8\text{-azido-ADP}\) is provided directly in the presence of Vi. The possible explanations for this apparently paradoxical result are considered under “Discussion.” In addition, Table I lists the activation energies for the basal and substrate (verapamil)-stimulated hydrolysis of ATP and 8-azido-ATP by Pgp, the Vi-induced trapping of \([\alpha^{32}P]8\text{-azido-ADP}\) under hydrolysis and non-hydrolysis conditions, and for the binding of \([\alpha^{32}P]8\text{-azido-ADP}\) and IAAP to Pgp. The activation energies for the hydrolysis of ATP both in the absence (basal) and in the presence of verapamil are comparable (115.5 and 110.4 kJ/mol) and almost identical to that for the hydrolysis of 8-azido-ATP (100.1 kJ/mol). In addition, the activation energy for ATP hydrolysis by Pgp in crude membranes derived from the Chinese hamster ovary cell line, CHB30 (98.1 kJ/mol) (35), is comparable to that obtained with human Pgp in this study. These data indicate that in terms of the thermodynamics of the system, ATP hydrolysis by Pgp is independent of the species origin of the protein or the nucleotide (ATP or 8-azido-ATP) and that the drug substrate does not significantly affect the activation energy for nucleotide hydrolysis. Even more intriguing is the fact that the activation energy for the Vi-induced trapping of \([\alpha^{32}P]8\text{-azido-ADP}\) under hydrolysis conditions is 62 kJ/mol or approximately half that for the substrate-stimulated ATP hydrolysis. The Vi-induced trapping of \([\alpha^{32}P]8\text{-azido-ADP}\) arrests the catalytic cycle after only one hydrolysis event and thus would have an activation energy one-half of that for the entire catalytic cycle. It is also clear from the data in Table I that the activation energies for binding of nucleoside diphosphate or substrate (IAAP) are significantly lower when compared with trapping of nucleotides. Also, whereas either \([\alpha^{32}P]8\text{-azido-ADP}\) or \([\alpha^{32}P]8\text{-azido-ATP}\) can be used to initiate Vi-induced trapping with very similar kinetics and functional effects, the energy barriers that these two pathways entail are significantly different (152 versus 62 kJ/mol).
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DISCUSSION

Due to the importance of Pgp in cancer chemotherapy and as a model system for ABC transporters in general, the catalytic cycle of this transporter has been studied in considerable detail (for reviews see Refs. 2, 14, and 30). Building upon the model proposed by Senior’s group (14), we have recently elucidated the properties of the transition state intermediate of Pgp initiated by using [\(\alpha^{32}\text{P}\)]8-azido-ADP trapping when the nucleoside diphosphate is provided directly to Pgp in the presence of Vi (Fig. 4, A and B). These results provide evidence that [\(\alpha^{32}\text{P}\)]8-azido-ADP is trapped into Pgp in the presence of Vi in a similar manner under both hydrolysis and non-hydrolysis conditions. In addition, [\(\alpha^{32}\text{P}\)]8-azido-ADP can be used to delineate the relationship between the two ATP sites in hydrolysis and Vi-induced trapping. Earlier work (6, 9) has shown that mutations in the conserved Walker B consensus motif in either the N- or the C-terminal halves of Pgp (9, 12, 27–29). Fig. 2 demonstrates that there is an almost equal distribution of [\(\alpha^{32}\text{P}\)]8-azido-ADP into the Pgp in the presence of Vi, [\(\alpha^{32}\text{P}\)]8-azido-ADP was trapped into the N- and C-terminal halves of Pgp (9, 12, 27–29). Fig. 2 demonstrates that Mg\(^{2+}\) or another divalent cation such as Mn\(^{2+}\) or Co\(^{2+}\) are necessary for ATP hydrolysis (31) and Vi-induced trapping of [\(\alpha^{32}\text{P}\)]8-azido-ADP (27, 31). Consistent with these findings, the data in Fig. 3 demonstrate that Mg\(^{2+}\), Mn\(^{2+}\), and Co\(^{2+}\) permit Vi-induced trapping of [\(\alpha^{32}\text{P}\)]8-azido-ADP under both hydrolysis and non-hydrolysis conditions.

It is important to determine whether the kinetics of Vi-induced trapping differs under hydrolysis and non-hydrolysis conditions. We show that during the trapping of [\(\alpha^{32}\text{P}\)]8-azido-ADP via the hydrolysis of [\(\alpha^{32}\text{P}\)]8-azido-ATP into Pgp, the \(K_m\) for [\(\alpha^{32}\text{P}\)]8-azido-ATP is nearly identical to the \(K_m\) for [\(\alpha^{32}\text{P}\)]8-azido-ATP trapping when the nucleoside diphosphate is provided directly to Pgp in the presence of Vi (Fig. 4, A and B). These results provide evidence that [\(\alpha^{32}\text{P}\)]8-azido-ADP is trapped into Pgp in the presence of Vi in a similar manner under both hydrolysis and non-hydrolysis conditions. In addition, [\(\alpha^{32}\text{P}\)]8-azido-ADP can be used to delineate the relationship between the two ATP sites in hydrolysis and Vi-induced trapping. Earlier work (6, 9) has shown that mutations in the conserved Walker B consensus motif in either the N- or the C-terminal ATP sites of Pgp arrest ATP hydrolysis as well as the Vi-induced trapping of [\(\alpha^{32}\text{P}\)]8-azido-ADP generated through the hydrolysis of [\(\alpha^{32}\text{P}\)]8-azido-ATP. The mutations in either the N-terminal ATP site (D555N) or the C-terminal site (D1200N) abolish Vi-induced trapping of [\(\alpha^{32}\text{P}\)]8-azido-ADP by both the hydrolysis and non-hydrolysis routes (Fig. 6). These findings demonstrate that both ATP sites are required not only for ATP hydrolysis but also for Vi-induced nucleoside diphosphate trapping even under non-hydrolysis conditions. Thus, except for binding of nucleotide, each ATP site does not appear to be able to carry out subsequent steps in the catalytic cycle.

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Properties of the Transition State Intermediate of Pgp

TABLE I

| Reaction | Temperature range | $E_{act}$ [kJ/mol] | $r$ |
|----------|-------------------|--------------------|-----|
| ATP hydrolysis (basal) | 22–39 | 115.5 | 0.993 |
| ATP hydrolysis (verapamil-stimulated) | 22–39 | 110.4 | 0.995 |
| 8-Azido-ATP hydrolysis (verapamil-stimulated) | 22–39 | 100.1 | 0.986 |
| Trapping, $[\alpha^{-32}P]8$-azido-ATP + Vi | 22–39 | 61.93 ± 10.25 | 0.929 |
| Trapping, $[\alpha^{-32}P]8$-azido-ADP + Vi | 22–39 | 151.98 ± 5.20 | 0.976 |
| Binding, $[\alpha^{-32}P]8$-azido-ADP | 22–39 | 19.50 | 0.994 |
| Binding, IAAP | 22–39 | 7.97 | 0.167 |

$^a$ Activation energy ($E_{act}$) was calculated from the slope of the Arrhenius plots similar to those depicted in Fig. 9B. $E_{act} = -(slope)/2.3R; R = 1.98$.

$^b$ Vi-sensitive basal (in the absence of drug substrate). ATP hydrolysis was measured as described below.

$^c$ Verapamil (30 μM)-stimulated ATP- and 8-azido-ATP hydrolysis by Pgp in crude membranes was carried out as described under “Experimental Procedures” at different temperatures ranging from 22 to 39 °C.

$^d$ Experiment was carried out as described in the legend to Fig. 9B (values represent mean ± S.D.; n = 3, and r is given for a representative experiment).

$^e$ Crude membranes (1 mg/ml) were incubated in the ATPase assay buffer with 10 μM $[\alpha^{-32}P]8$-azido-ATP (10 μCi/nmol) in the dark for 6 min at different temperatures ranging from 22 to 39 °C. Following UV irradiation and SDS-PAGE, the radioactivity incorporated in the Pgp band was determined using the PhosphorImager as described under “Experimental Procedures.” Values represent average of two experiments, and r is given for a representative experiment.

$^f$ Crude membranes (10–50 μg of protein) were incubated in 50 mM Tris-HCl, pH 7.5, with IAAP (5–7 nM) for 5 min under subdued light at different temperatures ranging from 22 to 39 °C. The samples were photocross-linked and, following SDS-PAGE radioactivity in the Pgp band, determined as described under “Experimental Procedures.” Values represent average of two experiments, and r is given for a representative experiment.

![Diagram](http://www.jbc.org/)

**FIG. 10. Activation energies ($E_{act}$) required for various steps in the catalytic cycle of ATP hydrolysis by Pgp.** The arrows as indicated represent the values (kJ/mol) for the activation energies of nucleotide binding (empty arrow); Vi-induced trapping of $[\alpha^{-32}P]8$-azido-ADP through hydrolysis of $[\alpha^{-32}P]8$-azido-ATP (gray arrow); hydrolysis of ATP or 8-azido-ATP in the presence or absence of drug-substrate verapamil (hashed arrow); and Vi-induced $[\alpha^{-32}P]8$-azido-ADP trapping in the absence of hydrolysis (dark arrow). These data are from Fig. 9B and Table I, and only average or mean values are given here. See legend to Fig. 2A for the description of the catalytic scheme and the text for details.

ATP in the presence of Vi under ATP hydrolysis conditions, the activation energy is 62 kJ/mol (mean value of three experiments). Conversely, when $[\alpha^{-32}P]8$-azido-ADP is directly trapped in the presence of Vi without ATP hydrolysis, the activation energy is 152 kJ/mol (mean value of three experiments). Thus, the latter route of generating the transition state conformation has an energy barrier ~2.5 times higher than the hydrolysis route. Moreover, the trapping of $[\alpha^{-32}P]8$-azido-ADP under non-hydrolysis conditions has an activation energy 1.5 times higher than that required for basal or verapamil-stimulated hydrolysis of ATP or 8-azido-ATP, which represents the complete catalytic cycle (see Table I and Fig. 10). This result is consistent with the hypothesis that the hydrolysis of $[\alpha^{-32}P]8$-azido-ATP provides energy to facilitate the conformational changes that accompany Vi-induced trapping. On the other hand, when $[\alpha^{-32}P]8$-azido-ADP is directly trapped into Pgp, there is no accompanying hydrolysis, and thus this reaction would necessarily have a much greater energy barrier to overcome; for this reason, in the normal catalytic cycle of ATP hydrolysis, this reaction would be highly unfavorable (see Fig. 10 for the comparison of the activation energies required for various steps in the catalytic cycle of ATP hydrolysis). These
data also provide an explanation for previous studies (3), which show that the inhibition of ATPase activity at 37 °C was much more rapid with ATP and Vi than with ADP and Vi. Moreover, as shown in Table I, the activation energy for the binding of \([\alpha-32P]8\text{-azido-ADP}\) in the absence of Vi is only 19.5 kJ/mol. This is about 7.5-fold lower than the activation energy for the trapping of \([\alpha-32P]8\text{-azido-ADP}\) in the presence of Vi. This suggests that it is not the binding step but the subsequent conformational changes that generate Vi-trapped intermediate(s) that are energetically intensive. Similarly, the activation energy for the binding of the hydrophobic drug-substrate IAAP to Pgp is extremely low (7.97 kJ/mol).

In recent years, the crystal structures of the ATP subunits of several ABC and analogous transporters have been resolved. These include the following: HisP, the ATP subunit of the bacterial histidine permease (36); MutS, a protein that recognizes mispaired and unpaired bases in duplex DNA and initiates mismatch repair (37, 38); ArsA, the soluble ATPase component of the bacterial arsenite pump, ArsAB (39); and MalK, the ATPase subunit of the trehalose/maltose transporter (40). In most of these studies (viz. MutS, ArsA, and MalK), the nucleoside diphosphate was directly incorporated by including ADP (and in some cases Vi or aluminum fluoride) during crystallization. Our study suggests that these structures where the ADP has not been incorporated in situ by the hydrolysis of ATP are nonetheless representative of the native conformation.

Also, published reports postulate that the nucleoside diphosphate may have interesting regulatory roles to play in the catalytic cycles of several ABC transporters such as cystic fibrosis transmembrane conductance regulator and the sulfonamide receptor, SUR1 (41–43). In many of these instances (32), the activation energy for the binding of \([\alpha-32P]8\text{-azido-ADP}\) would prove very useful in designing experimental strategies to address these hypotheses directly.

Taken together, our results provide compelling evidence that although, there is a 2.5-fold difference in the activation energies required to generate the Pgp-[\alpha-32P]8-azido-ADP-Vi complex using [\alpha-32P]8-azido-ADP and Vi compared with [\alpha-32P]8-azido-ATP and Vi, the transition state complex generated by either route is functionally indistinguishable. Our preliminary observations with another ABC transporter, the MRP1 (44), suggest that MRP1 also exhibits Vi-induced trapping of [\alpha-32P]8-azido-ADP under both hydrolysis and non-hydrolysis conditions. These findings are consistent with the results reported in this paper for Pgp. This work, however, does not address the effect of drug substrates on Vi-induced trapping of [\alpha-32P]8-azido-ADP under non-hydrolysis conditions. These experiments are currently in progress.

**Acknowledgments**—We thank Dr. Michael M. Gottesman for discussions, encouragement, and for critical comments on the manuscript. We also thank Drs. Christine A. Hrycyna and Michael M. Gottesman for providing the wild-type and mutant MDR1 constructs.

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\*M. Muller, M. M. Smith, Z. E. Sauna, and S. V. Ambudkar, unpublished observations.

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Zuben E. Sauna, Melissa M. Smith, Marianna Müller and Suresh V. Ambudkar

J. Biol. Chem. 2001, 276:21199-21208.
doi: 10.1074/jbc.M100886200 originally published online April 3, 2001

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