Characterization of the Catalytic Cycle of ATP Hydrolysis by Human P-glycoprotein

THE TWO ATP HYDROLYSIS EVENTS IN A SINGLE CATALYTIC CYCLE ARE KINETICALLY SIMILAR BUT AFFECT DIFFERENT FUNCTIONAL OUTCOMES

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P-glycoprotein (Pgp) is a plasma membrane protein whose overexpression confers multidrug resistance to tumor cells by extruding amphipathic natural product cytotoxic drugs using the energy of ATP. An elucidation of the catalytic cycle of Pgp would help design rational strategies to combat multidrug resistance and to further our understanding of the mechanism of ATP-binding cassette transporters. We have recently reported (Sauna, Z. E., and Ambudkar, S. V. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2515–2520) that there are two independent ATP hydrolysis events in a single catalytic cycle of Pgp. In this study we exploit the vanadate (Vi)-induced transition state conformation of Pgp (Pgp-ADP-Vi) to address the question of what are the effects of ATP hydrolysis on the nucleotide-binding site. We find that at the end of the first hydrolysis event there is a drastic decrease in the affinity of nucleotide for Pgp coincident with decreased substrate binding. Release of occluded dinucleotide is adequate for the next hydrolysis event to occur but is not sufficient for the recovery of substrate binding. Whereas the two hydrolysis events have different functional outcomes vis-à-vis the substrate, they show comparable $k_h$ for both incorporation and release of nucleotide, and the affinities for [γ-32P]8-azido-ATP are identical. In addition, the incorporation of [γ-32P]8-azido-ADP in two ATP sites during both hydrolysis events is also similar. These data demonstrate that during individual hydrolysis events, the ATP sites are recruited in a random manner, and only one site is utilized at any given time because of the conformational change in the catalytic site that drastically reduces the affinity of the second ATP site for nucleotide binding. In aggregate, these findings provide an explanation for the alternate catalysis of ATP hydrolysis and offer a mechanistic framework to elucidate events at both the substrate- and nucleotide-binding sites in the catalytic cycle of Pgp.

Expression of the plasma membrane protein, P-glycoprotein (Pgp), confers multidrug resistance to tumor cells. It belongs to the ATP-binding cassette (ABC) superfamily of transport proteins, characterized by two homologous halves containing six transmembrane helices and one nucleotide-binding site in each half (1, 2). Pgp confers drug resistance to tumor cells by extruding cytotoxic natural product hydrophobic drugs using the energy of ATP hydrolysis (2, 3). Pgp interacts not only with nucleotides (4–6) and cytotoxic drugs (3) but also with a diverse set of other lipophilic compounds (7). Additionally, besides the transport of these compounds coupled to ATP hydrolysis, other complex interactions are known to occur in Pgp with modulators of the multidrug resistance (MDR) phenotype (8). Considering the importance of Pgp in cancer chemotherapy and as a model system for ABC transporters in general, a clear understanding of the catalytic cycle of this transporter is of considerable importance.

The first catalytic scheme proposed for Pgp (9) was that of the ATP hydrolysis reaction. The essential feature of this model is alternating hydrolysis of ATP at the two ATP-binding sites. It was postulated that nucleotide first binds to one of the two sites but could not be hydrolyzed. When another nucleotide binds to the second site it promotes hydrolysis at the first site, which in turn powers substrate transport. In the next cycle, hydrolysis occurs at the second ATP site. We have recently (10) demonstrated that there is substantially greater complexity in the catalytic cycle of Pgp. We simultaneously monitored changes in the substrate- and nucleotide-binding sites to show that although binding of nucleotide per se does not affect interactions with the substrate, ATP hydrolysis results in a dramatic conformational change where the affinity of the substrate $[\text{Iodoarylazidoprazosin} ([\text{IAP})$ for Pgp trapped in the transition state conformation (Pgp-ADP-Vi) is reduced >30-fold. Even more remarkable is the finding that to transform Pgp from this intermediate state of low affinity for substrate to the next catalytic cycle, i.e. a conformation that binds substrate with high affinity, the hydrolysis of an additional molecule of nucleotide is obligatory. This requirement for two ATP hydrolysis events in the course of a single catalytic cycle was consistent with the measured stoichiometry for Pgp where two ATP molecules are hydrolyzed for each substrate molecule transported (11, 12).

In this study we have analyzed, in real time, the repeating succession of vanadate (Vi)-induced trapping and release of [γ-32P]8-azido-ADP through an entire catalytic cycle, and we monitored the substrate ($[\text{Iodoarylazidoprazosin} ([\text{IAP})$ binding at the beginning...
and end of each trapping (hydrolysis) event. The results, besides validating our model, provided experimental strategies for independently studying the kinetics of the two hydrolysis events. We find that at the end of the first hydrolysis event there is a >30-fold decrease in the affinity of nucleotide for Pgp coincident with the decreased substrate binding shown earlier (10, 13). Release of occluded ADP or 8-azido-ADP is adequate for the next hydrolysis event to occur but is not sufficient for recovery of substrate binding, which occurs only at the end of the second hydrolysis, i.e. after ATP dissociates from the transporter. Whereas the two hydrolysis events have different functional outcomes vis à vis the substrate, they show comparable $t_1/2$ for both incorporation and release of nucleotide, and the $K_m$ values for [α-32P]8-azido-ATP during the Vi-induced trapping during both hydrolysis events are identical. Similarly, the incorporation of [α-32P]8-azido-ADP in both the N-terminal and the C-terminal ATP sites is identical during both ATP hydrolysis events. These data demonstrate that during individual hydrolysis events the ATP sites are recruited in a random manner, and the fact that only one site is utilized at any given time is a consequence of the conformational change that reduces the affinity of nucleotide for the second ATP site. Thus, the prevention of ATP binding to the second site while the first one is in a catalytic conformation appears to be the explanation for alternate catatlysis in Pgp.

**Experimental Procedures**

Chemicals—Cyclosporin A was purchased from Calbiochem, and [125I]lodoarylazidoprazosin ([125I]IAAP) (2, 200 Ci/mmol) was obtained from PerkinElmer Life Sciences. [α-32P]8-Azido-ATP (15–20 Ci/mmole) and 8-azido-ATP were purchased from Affinity Labeling Technologies, Inc (Lexington, KY). TNP-ATP was procured from Molecular Probes (Eugene OR). 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 (H6)) as described (13). Crude membranes were prepared as described previously (4, 13).

Purification of Pgp—Human Pgp was purified as described previously with minor changes. Briefly, crude Pgp-containing membranes were solubilized with octyl-$\beta$-D-glucopyranoside (1.25%) in the presence of 20% glycerol and 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-$\beta$-D-glucopyranoside and 0.04% lipid; 80% purified Pgp was eluted with 100 mM imidazole. Pgp in 100 mM imidazole fraction was then concentrated (Centriprep-50, Amicon, Beverly, MA) to ~0.5 mg/ml and stored at 70 °C.

8-Azido-ATP was a useful photoaffinity reagent to understand its interactions with the substrate, similarly Fig. 1 demonstrates that the binding of the nucleotide, [α-32P]8-azido-ATP, is not affected by substrates such as verapamil and cyclosporin A. Earlier work from our laboratory (10, 13) demonstrated that

**FIG. 1.** Effect of substrates on the binding of [α-32P]8-azido-ATP to Pgp.

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**Results**

**The Vi-induced ADF-trapped Conformation of Pgp during ATP Hydrolysis Exhibits a Marked Decrease in Affinity for Both Drug Substrate [125I]IAAP and [α-32P]8-Azido-ATP—8-Azido-ATP is a good hydrolysis substrate for both Chinese hamster and human Pgp, with a $K_m$ similar to ATP (6, 15, 16).** – Labeled 8-azido-ATP is a useful photoaffinity reagent to understand the nucleotide binding and ATP hydrolysis events that accompany drug-substrate transport in Pgp. Our recent studies (10) show that binding of nucleotide to Pgp per se does not affect its interactions with the substrate, similarly Fig. 1 demonstrates that the binding of the nucleotide, [α-32P]8-azido-ATP, is not affected by substrates such as verapamil and cyclosporin A.
The Vi-trapped conformation of Pgp binds substrates with at least 30-fold reduced affinity. The data in Fig. 2A show that if the photoaffinity substrate analogue, [125I]IAAP, is allowed to bind Pgp, which has been treated previously with increasing concentrations of 8-azido-ATP in the presence of Vi at 37 °C (which corresponds to increased trapping of 8-azido-ADP in Pgp), there is a concentration-dependent decrease in substrate binding. A single phase exponential decay model fits the data with a $K_i = 18 \mu M$ for the 8-azido-ATP inhibition of [125I]IAAP binding. It is also clear from these data that for maximal trapping of 8-azido-ADP in the presence of Vi, 0.5 to 1 mM 8-azido-ATP is required. Our earlier work (10) and data in Fig. 3A suggest that the binding of nucleotide first binds to one of the two ATP sites precludes ATP hydrolysis (3, 6, 11). The literature that there is communication between the two ATP sites precludes ATP hydrolysis (3, 6, 11). The compound exhibits a low quantum yield in an aqueous medium with an emission maxima at 550 nm (excitation 408 nm), and an increase in quantum yield accompanied by a small blue shift (new emission maxima at 530 nm) occurs when TNP-ATP binds to the ATP sites of Pgp (19). However, when Pgp is pretreated with Vi and 8-azido-ATP at 37 °C to generate the Pgp-8-azido-ADP-Vi complex, there is a marked decrease in the fluorescence (Fig. 3A) suggesting that the binding of nucleotide (TNP-ATP) to Pgp is drastically reduced, consistent with the results obtained in the experiments with [32P]-8-azido-ATP (Fig. 2, B and C).

The viability of these experiments is that the reduced binding of nucleotide to Pgp in the presence of ATP and Vi is depicted in Fig. 3B. Purified, reconstituted Pgp was treated with increasing concentrations of ATP in the presence of Vi at 37 °C for 10 min, and excess ATP and Vi were removed by centrifugation. Extent of TNP-ATP binding to Pgp was quantified by measuring the fluorescence intensity (Ex = 408 nm and Em = 530 nm). A single phase exponential decay model fits the data with a $K_i$(ATP) of 83 μM. Dissociation of [32P]-8-Azido-ADP from the Pgp-8-Azido-ADP-Vi Complex Is Not Affected by Nucleotides—The results thus far show that following ATP hydrolysis, affinity for nucleotide is considerably reduced. There is considerable evidence in the literature that there is communication between the two ATP sites of Pgp and that mutations or chemical modification of residues in either ATP site reduces ATP hydrolysis (3, 6, 15, 20–25). This has led to the hypothesis that there is alternating hydrolysis of ATP at the two ATP sites (9, 26, 27), i.e. that nucleotide first binds to one of the two sites but cannot be hydrolyzed. When another nucleotide binds to the second ATP site it promotes hydrolysis at the first site, which in turn powers substrate transport. In the context of our results this raises the following two questions. (a) Does nucleotide binding per se affect Vi-induced trapping? (b) Once ATP hydrolysis
ensues, do nucleotides influence the release of ADP from Pgp? To address the first question, we monitored Vi-induced trapping of [α-32P]8-azido-ADP in the presence increasing concentrations of AMPPNP, a nucleotide analogue, that can bind to Pgp but cannot be hydrolyzed (10). Thus, if binding of nucleotide at one ATP site exerts allosteric control over hydrolysis at the other ATP site, binding of AMPPNP at one site should stimulate Vi-induced trapping at the other site. Fig. 4A, however, clearly demonstrates that AMPPNP inhibits in a dose-dependent manner the Vi-induced trapping of [α-32P]8-azido-ADP with an apparent K_i = 1.3 mM. This suggests that binding of nucleotide at one site does not stimulate ATP hydrolysis at the other site.

To address the second question, i.e. what controls the release of ADP from Pgp after ATP hydrolysis, we monitored the dissociation of [α-32P]8-azido-ADP from the Pgp-8-azido-ADP-Vi complex. The [α-32P]8-azido-ADP was trapped by incubating crude membranes with [α-32P]8-azido-ATP and Vi at 37 °C for 10 min, and free Vi and nucleotide were removed by centrifugation, and the resuspended membranes were incubated at 37 °C under different conditions. Aliquots were removed at intervals, and excess cold ATP was added to displace the [α-32P]8-azido-ADP dissociated from the complex, and the samples were cross-linked by UV irradiation. Fig. 4B shows that the [α-32P]8-azido-ADP is completely dissociated within 15 min at 37 °C with a t_1/2 of 2–3 min. The t_1/2 for dissociation is not influenced by the presence of excess 8-azido-ADP, 8-azido-ATP, or the nonhydrolyzable nucleotide AMPPNP, added in the presence of Mg^{2+}. The dissociation is, however, strongly temperature-dependent with no appreciable dissociation during a 15-min incubation at room temperature (data not shown). These results suggest that following ATP hydrolysis, ADP dissociates spontaneously and that while in the transition state, ADP or 8-azido-ADP is occluded and not exchangeable with exogenously added nucleotide.

Characterization of the Two ATP Hydrolysis Events in a Single Catalytic Cycle of Pgp—The experiments described above demonstrate that the ATP hydrolysis that follows binding of nucleotide and substrate results in a conformational change in Pgp that dramatically lowers the affinity of both substrate and nucleotide and inhibits the binding of additional molecules. Fig. 5A depicts an experimental strategy, described in detail in the legend, to discriminate between the two hydrolysis events postulated to occur in a single catalytic cycle of Pgp (10). Essentially, Pgp can be put through repeating cycles of Vi-induced [α-32P]8-azido-ADP trapping and release, and the kinetic parameters for each step can be determined. In Fig. 5B we depict the rate constants for the repeating cycles of Vi-induced [α-32P]8-azido-ADP trapping and release. The data show that the rate constants for two hydrolysis events (cf. Fig. 5B) are indistinguishable. The occurrence of two ATP hydrolysis events in a single catalytic cycle raises the question, is there a functional asymmetry between the two ATP sites? For example, is the N-terminal ATP site committed to the first hydrolysis event followed by the recruitment of the C-terminal ATP site for the second ATP hydrolysis event? We addressed this question by performing a mild trypsin digestion of Pgp after labeling with [α-32P]8-azido-ADP at steps II, IV, and VI of the catalytic cycle, as depicted in Fig. 5A. The results (Fig. 5C) clearly demonstrate that at high concentration of [α-32P]8-azido-ATP, there is no preference for either the N- or the C-terminal ATP site during any of the ATP hydrolysis events.

Binding of [125I]IAAP to Pgp at Various Steps in the Catalytic Cycle of ATP Hydrolysis—We postulated earlier that two hydrolysis events occur during a single cycle of Pgp (10) based on the following evidence: (a) following ATP hydrolysis Pgp takes on a conformation with >30-fold reduced affinity for substrate, and (b) for Pgp to regain the conformation that binds substrate with high affinity a second ATP hydrolysis event is mandatory. Although Fig. 5B tracks ATP hydrolysis over the entire catalytic cycle and is consistent with our previous findings, it does not explicitly demonstrate the status of the substrate-binding site prior to and following each hydrolysis event. Fig. 6 shows [125I]IAAP binding to Pgp at different steps in the catalytic cycle as depicted in Fig. 5B, which, re-plotted on a continuous time scale, is shown as a gray background. The gray area thus represents the extent of [α-32P]8-azido-ADP incorporation and release in a single catalytic cycle, and the filled bars depict the extent of [125I]IAAP incorporated into Pgp at various steps in the catalytic cycle (see Fig. 5, A and B). The data for [α-32P]8-azido-ADP incorporation and release, which have been quantitatively treated in Fig. 5B, merely offer a visual representation in Fig. 6 of the state of Pgp molecules in a single catalytic cycle.

Prior to the first hydrolysis event corresponding to the zero time point in Fig. 5A (t), there is a normal level of [125I]IAAP binding (Fig. 6, step I), similar to untreated Pgp. Immediately

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**Fig. 3.** The Pgp-8-azido-ADP-Vi transition state complex shows reduced binding of the fluorescent nucleotide TNP-ATP. A. TNP-ATP binding to Pgp in transition state conformation generated by pretreatment with 8-azido-ATP and Vi. Purified Pgp was reconstituted into liposomes as described previously (16). Proteoliposomes (25 μg of protein) were incubated with 100 μM TNP-ATP at room temperature for 10 min, and emission scans were obtained (excitation = 408 nm; emission = 500–600 nm). Scans taken in the presence of Pgp were corrected for nonspecific binding by subtracting scans acquired in the presence of 100-fold excess ATP. Figure shows TNP-ATP + Pgp, control (continuous line) and TNP-ATP + Pgp pre-trapped with 8-azido-ADP and Vi (dashed line). Pgp was trapped with 8-azido-ATP by incubating with 8-azido-ATP, 1.25 mM in the presence of Vi, 0.25 mM for 10 min at 37 °C. The reaction was stopped by the addition of 10 mM ice-cold ATP, and excess nucleotides and Vi were removed by centrifugation. B, kinetics of inhibition of TNP-ATP binding to Pgp pretreated with ATP and Vi. Proteoliposomes containing purified Pgp (20–25 μg) were treated with increasing concentrations of ATP and Vi (250 μM) at 37 °C for 10 min. Excess ATP and Vi were removed by centrifugation at 300,000 × g at 4 °C for 10 min. The membranes were resuspended in ATPase assay buffer and incubated with 100 μM TNP-ATP at room temperature for 10 min, and emission scans were obtained (excitation = 408 nm; emission = 500–600 nm). Scans taken in the presence of Pgp were corrected for nonspecific binding by subtracting scans acquired in the presence of 100-fold excess ATP. The peak fluorescence signal at 530 nm for TNP-ATP at each concentration of ATP in the presence of Vi was determined, and the data were normalized to the fluorescence of the sample with no ATP. The data were fitted using GraphPad Prism 2.0 for the PowerPC Macintosh.
The Two Hydrolysis Events in a Single Catalytic Cycle of Pgp Have Identical \( K_m \) Values for \(^{32}\text{P}\) 8-azido-ATP in the Presence of Vi—To obtain the \( K_m \) of \(^{32}\text{P}\) 8-azido-ATP for Pgp during Vi-induced trapping, crude membranes containing Pgp were incubated with increasing concentrations of \(^{32}\text{P}\) 8-azido-ATP in the presence of Vi at 37 °C. The reaction was stopped with excess ATP. \(^{32}\text{P}\)-Azido-ATP incorporated into Pgp was quantified from a SDS-PAGE gel as described under "Experimental Procedures." The extent of \(^{32}\text{P}\) 8-azido-ATP trapping in the first hydrolysis event is depicted in Fig. 7A. The single site model of Henri-Michaelis-Menten best described the data to give a \( K_m \) of 18.4 μM. To determine the kinetics of the second hydrolysis event, the crude membranes were first allowed to trap 8-azido-ATP in the presence of Vi for 10 min; excess 8-azido-ATP and Vi were removed by centrifugation, and then the occluded 8-azido-ATP was allowed to dissociate by incubation at 37 °C for 15 min (steps I and II in Fig. 5A). The second hydrolysis event was followed in the presence of increasing concentrations of \(^{32}\text{P}\) 8-azido-ATP and Vi, and the extent of \(^{32}\text{P}\) 8-azido-ATP incorporation was quantified as described above. The data (Fig. 7B) show that the \( K_m \) value for \(^{32}\text{P}\) 8-azido-ATP in the presence of Vi during the second hydrolysis event, 19.4 μM, is indistinguishable from that for the first hydrolysis event. However, the results depicted in Fig. 7 substantiate the notion that although the hydrolysis events measured in Figs. 7, A and B, are kinetically similar, they occur in two distinct conformations of Pgp vs à vs the substrate binding (Figs. 5B and 6).

**DISCUSSION**

Our recent work has demonstrated that there are two independent ATP hydrolysis events in a single catalytic cycle of Pgp. In this study, we exploit the vanadate (Vi)-induced transition state conformation of Pgp (Pgp-ADP-Vi) to address the question of what are the effects of ATP hydrolysis on the nucleotide-binding site. We also track, in real time, the repeating succession of trapping and release of \(^{32}\text{P}\) 8-azido-ADP through an entire catalytic cycle, and we independently study the kinetics of the two hydrolysis events. Crude membrane fractions as well as purified Pgp reconstituted into lipid vesicles manifest a basal level of ATP hydrolysis that is stimulated by substrates and modulators of Pgp by a factor of up to 10 (3, 9). Pgp exhibits low affinity for ATP (\( K_m \), 0.3 to 1 mM) compared with, for example, myosin or the mitochondrial \( F_0F_1 \)-ATP synthase (28, 29). Additionally, in Pgp no covalent phosphorylated (E-P) intermediate has been demonstrated as is known to occur for the P-type ATPases (9). These facts led Senior and coworkers (9, 26) to hypothesize that during ATP hydrolysis a state of high chemical potential is generated and that the relaxation of such a state powers the extrusion of substrate.

Recent work from our laboratory (10) experimentally demonstrated a large conformational change accompanying ATP hydrolysis. We showed that the long lived Pgp-8-azido-ADP-Vi transition state complex, which is generated immediately following ATP hydrolysis, exhibits a drastic decrease in the affinity for the substrate analogue \(^{32}\text{P}\) IAAAP. Even more interesting was the observation that the release of the occluded 8-azido-ATP was not sufficient to regain substrate binding, which occurred only after an additional ATP hydrolysis step. That two hydrolysis events occur in a single catalytic cycle, one associated with efflux of drug and the other to bring about conformational changes that "reset" the molecule, raise several mechanistic questions about ATP hydrolysis during the catalytic cycle of Pgp. Is there conformational coupling between the drug-binding sites and the ATP sites, and by extension what is the status of the drug and nucleotide binding at each step in the catalytic cycle? What effect, if any, does ATP hydrolysis have on

Following ATP hydrolysis, i.e., the final time point in Fig. 5A (step I) or the first time point in Fig. 5A (step II), the \(^{32}\text{P}\) IAAAP binding is reduced by >90% (Fig. 6, step II). Following release of occluded 8-azido-ADP, in the absence of ATP hydrolysis (the final time point in Fig. 5A (step II)), \(^{32}\text{P}\) IAAAP binding continues to be reduced (Fig. 6, step III). Vi-induced trapping of 8-azido-ADP in the second cycle (Fig. 5A (step III)) does not show any recovery of \(^{32}\text{P}\) IAAAP binding (Fig. 6, step IV). However, instead of trapping 8-azido-ADP, if hydrolysis is allowed to occur in the absence of Vi, there is full recovery of \(^{32}\text{P}\) IAAAP binding (Fig. 6, step V). Similarly, release of the occluded 8-azido-ADP following the second hydrolysis (Fig. 5A (step VI)) allows recovery of \(^{32}\text{P}\) IAAAP binding (Fig. 6, step V). Taken together with Fig. 5B, these results further support the model we have previously proposed (10) and establish that two kinetically similar but functionally different ATP hydrolysis events are occurring in a single catalytic cycle.

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**Fig. 4. Effect of nucleotides on the Vi-induced trapping of \(^{32}\text{P}\) 8-azido-ATP and on the dissociation of \(^{32}\text{P}\) 8-azido-ADP from the Pgp-8-azido-ADP-Vi complex.** A, effect of the nonhydrolyzable nucleotide AMPPNP on Vi-induced trapping of \(^{32}\text{P}\) 8-azido-ADP into Pgp. Crude membranes (1 mg/ml protein) were incubated in the dark at 37 °C for 10 min with 50 μM \(^{32}\text{P}\) 8-azido-ATP (3–5 μCi/nmol), 250 μM Vi, and increasing concentrations of AMPPNP in the ATPase assay buffer. The reaction was stopped by adding 12.5 mM ice-cold ATP and placing the tubes on ice. Following photo-cross-linking by UV irradiation at 365 nm for 5 min and SDS-PAGE, the level of \(^{32}\text{P}\) 8-azido-ADP incorporated was quantified using a PhosphorImager, and the data were fit by using GraphPad Prism 2.0 for the PowerPC Macintosh. A single phase exponential decay model best fits the data. B, nucleotides have no effect on the rate of dissociation of \(^{32}\text{P}\) 8-azido-ADP from the Pgp-8-azido-ADP-Vi complex. Crude membranes (1 mg/ml protein) were incubated in the dark at 37 °C for 10 min with 50 μM \(^{32}\text{P}\) 8-azido-ATP (3–5 μCi/nmol) and 250 μM Vi in the ATPase assay buffer. The reaction was stopped by adding 12.5 mM ice-cold ATP and placing the tubes on ice. Untrapped nucleotides and excess Vi were removed by centrifugation at 300,000 × g for 10 min, and the membranes were resuspended in the Mg\(^2+\)-free ATPase assay buffer and divided into 4 aliquots. To these were added 10 mM MgCl\(_2\) control membranes; 10 mM MgCl\(_2\) + 1.25 mM ATP; 10 mM MgCl\(_2\) + 1.25 mM AMPPNP; or 10 mM EDTA, respectively, and incubated at 37 °C. Aliquots were removed at indicated intervals and placed on ice and photo-cross-linked by UV irradiation at 365 nm for 5 min, and SDS-PAGE was performed on each sample. The level of \(^{32}\text{P}\) 8-azido-ADP incorporated was quantified using a PhosphorImager, and the data were fit using GraphPad Prism 2.0 for the PowerPC Macintosh. Dissociation of \(^{32}\text{P}\) 8-azido-ADP in control membranes (○) and in the presence of 1.25 mM ATP (△), 1.25 mM AMPPNP (■), or Mg\(^2+\)-free medium containing 10 mM EDTA (■) are shown in the figure.
Fig. 5. Repeating cycles of ATP hydrolysis. A, schematic representation of the experiment. Step I and II, crude membranes (protein, 1 mg/ml) were incubated in the dark at 37 °C with 50 μM [α-32P]8-azido-ATP (3–5 μCi/nmol) and 250 μM Vi in the ATPase assay buffer. Aliquots were removed at regular intervals over 15 min and placed on ice. The reaction was stopped by adding excess ice-cold ATP (12.5 mM) and photo-cross-linking by UV irradiation at 365 nm for 5 min on ice. Steps II and III, at the end of 15 min, the reaction mixture was transferred to ice, and un-trapped nucleotides and excess Vi were removed by centrifugation at 300,000 × g for 10 min. The membranes were resuspended in the ATPase assay buffer and placed at 37 °C. Aliquots were again removed at indicated intervals over the next 20 min (35 min from start of experiment), and photo-cross-linked as described above. Step III and IV, at the end of 35 min, fresh 50 μM [α-32P]8-azido-ATP and 0.25 mM Vi were added to initiate the next cycle of ATP hydrolysis. Aliquots were removed at regular intervals, over an additional 15 min (50 min from start of experiment) and photo-cross-linked as described above. At the end of 15 min the reaction mixture was transferred to ice, and un-trapped nucleotides and excess Vi were removed by centrifugation at 300,000 × g for 10 min, and the membranes were resuspended in the ATPase assay buffer and placed at 37 °C. Steps IV and V, the next cycle of dissociation of [α-32P]8-azido-ADP was monitored by placing the reaction mixture at 37 °C and removing aliquots as indicated above. Steps V and VI, at the end of 20 min (70 min from start of experiment) a third hydrolysis cycle was initiated by adding 50 μM [α-32P]8-azido-ATP and 0.25 mM Vi and followed over the next 10 min (80 min from start of experiment). At the end of the experiment, all samples were electrophoresed and the [α-32P]8-azido-ADP incorporated into the Pgp band quantified using a PhosphorImager. The data were fitted to a single phase exponential decay model and the t_1/2 was determined for each step. C, distribution of Vi-trapped [α-32P]8-azido-ADP in the two ATP sites. At steps II, IV, and VI (described in Fig. 4A), following photo-cross-linking, the samples were treated with trypsin (protein:trypsin, 10:1) for 5 min at 37 °C to separate

subsequent nucleotide binding? Do the two ATP hydrolysis events show different kinetics?

Implicit in the model for the catalytic cycle proposed by Senior and others (24, 26, 27) is long range conformational coupling between the drug- and nucleotide-binding sites. Although direct evidence for such a relationship was lacking, interactions such as the stimulation of ATPase activity by drugs (4, 5, 30) have been accepted as evidence for the structural interactions that underlie such a coupling (26, 27). Our recent work (10) and Fig. 2A on the other hand quantitatively corroborate that large changes occur in the substrate-binding site as a consequence of ATP hydrolysis. It is critical, however, to demonstrate that these effects are not coincidental. A comparison of the $K_i$ for Vi-induced trapping of $[\alpha-32P]8$-azido-ADP (Fig. 7A) with the $K_i$ (8-azido-ATP) for inhibition of $[125I]$IAAP binding during trapping (Fig. 2A) shows the two values to be almost identical ($K_i$, 22 $\mu M$; $K_i$, 18 $\mu M$), and a cause-effect relationship between the two is plausible. Moreover, the fact that Vi-induced trapping of 8-azido-ATP inhibits substrate ($[125I]$IAAP) binding in a concentration-dependent manner (Fig. 2A) provides stronger and more direct evidence for interactions between the substrate- and nucleotide-binding sites. That these are long range interactions resulting from conformational changes in the protein is underscored by the fact that drugs do not affect nucleotide binding per se (Fig. 1), nor do nucleotides affect substrate binding in the absence of ATP hydrolysis (10).

The consequences of ATP hydrolysis on substrate binding have been elucidated elsewhere (10) and are described briefly above. To understand the effect of ATP hydrolysis on the affinity of nucleotides for the ATP sites, we once more exploited the Vi-trapped, transition state complex (Pgp-ADP-Vi). Fig. 2C illustrates that if crude membranes containing Pgp are pretreated with 8-azido-ATP and Vi, they show reduced binding of $[\alpha-32P]8$-azido-ATP. The $K_i$ (8-azido-ATP) for inhibition of $[\alpha-32P]8$-azido-ATP binding (12.3 $\mu M$) is comparable to the $K_i$ ($[\alpha-32P]8$-azido-ATP) for Vi-induced trapping of $[\alpha-32P]8$-azido-ADP (22 $\mu M$) and the $K_i$ (8-azido-ATP) for inhibition of $[125I]$IAAP binding (19 $\mu M$). These data suggest that the conformational changes that follow ATP hydrolysis reduce the affinity of both substrate and nucleotide for Pgp and the extent of decrease in the affinity of the nucleotide; $[\alpha-32P]8$-azido-ATP (Figs. 2C and 3) is comparable to that demonstrated earlier for the substrate (10) $[125I]$IAAP. The release of $[\alpha-32P]8$-azido-ADP from the complex is spontaneous and not affected by nucleotide binding (Fig. 4A), and this is sufficient for the next ATP hydrolysis event to ensue (Fig. 5B). The conformational changes occurring in the ATP sites are thus different from those in the substrate site in that, except for the release of occluded ADP, there is no additional energetic requirement for resetting the nucleotide-binding pocket.

There is strong evidence to support the occurrence of two hydrolysis events in a single catalytic cycle. Useful information should be gained by studying these two events independently. Data depicted in Fig. 5B, which follows the experimental strategy depicted in Fig. 5A, show that it is experimentally possible...
to propel Pgp through repeating cycles of Vi-induced $[\alpha-32P]8$-azido-ADP trapping and $[\alpha-32P]8$-azido-ADP release. Although the $t_d$ for each trapping and release event are comparable, Fig. 6 shows how the conformational state of the molecule differs during each of these events. It is clear from the gray-shaded area in Fig. 6 (data shown in Fig. 5B) that the two hydrolysis events are indistinguishable. Concurrent measurements of substrate binding, however, distinguish different states of the Pgp molecule in which these two events occur. Thus, when the first hydrolysis event is initiated (Fig. 6, step I), $[125I]IAAP$ binding is not affected in contrast to over 90% inhibition of $[125I]IAAP$ binding when the second hydrolysis event is initiated (Fig. 6, step III). At the end of the second hydrolysis event, however, $[125I]IAAP$ binding is restored to normal levels (Fig. 6, step V) bringing Pgp to a state comparable to step I both in terms of substrate and nucleotide binding, suggesting the completion of one catalytic cycle. It would thus be reasonable to conclude that determining the kinetics of Vi-induced $[\alpha-32P]8$-azido-ADP trapping between steps I and II, as depicted in Fig. 5, would represent the first hydrolysis event and between steps III and IV the second event. Fig. 7 shows that the $K_m$ ($[\alpha-32P]8$-azido-ATP) for trapping is identical for the two hydrolysis events. Thus, even though these events have different functional consequences, they are kinetically indistinguishable. This would suggest that it is unlikely that the two hydrolysis events are individually associated with each ATP site. We propose that the nucleotide-binding site for any hydrolysis event at high nucleotide concentration is recruited randomly, that all hydrolysis events are kinetically equivalent, and that the different functional outcomes are a result of the conformational state of the Pgp molecule when a particular hydrolysis event occurs. This view is also consistent with the findings of Urbatsch et al. (31) that the two ATP sites are functionally equivalent. The fact that following Vi-induced trapping $[\alpha-32P]8$-azido-ADP is distributed equally in both N- and C-terminal ATP sites (Fig. 5C) at steps II, IV, and VI, as depicted in Fig. 5A further supports this view.

Earlier studies with plasma membranes from Chinese hamster ovary cells overexpressing Pgp clearly showed that the trapped $[\alpha-32P]8$-azido-ATP labels the two ATP sites in equal proportion (32, 33). Our results with recombinant human Pgp similarly demonstrate that this distribution remains constant regardless of whether it is monitored at step II, IV, or VI of the cycle as depicted in Fig. 5A (Fig. 5C). These results strongly favor the conclusion that the two ATP sites are recruited randomly and show similar kinetics, which emerge as a single $K_m$(ATP or 8-azido-ATP) during hydrolysis (6, 13, 15, 16). Moreover, the observation that trapping of 1 mol of ADP/mol of Pgp is sufficient to block ATP hydrolysis (34, 35) has been interpreted to mean that trapping of nucleotide at one site blocks catalysis at both sites. This observation has in turn led to the speculation (26, 27) that the binding of nucleotide at one ATP site is not sufficient for hydrolysis to occur and that the binding of nucleotide at the second ATP site permits hydrolysis at the first site by an allosteric mechanism. Data presented in this work, on the other hand, provide direct experimental evidence that following ATP hydrolysis the affinity of nucleotide for Pgp is drastically reduced (Figs. 2C and 3). Thus, the fact that trapping nucleotide at either ATP site blocks hydrolysis at both can be explained by this conformational change that drastically reduces the affinity of nucleotide for the second ATP site. The reduced affinity for nucleotides to Pgp in the transition state is also demonstrated by using the fluorescent nucleotide analogue, TNP-ATP (Fig. 3, A and B). Such a perspective would also be consistent with the characterization of ATP hydrolysis in Pgp (28) which shows Henri-Michaelis-Menten kinetics with a single $K_m$ in the 0.3–1 m M range, since no cooperativity has been demonstrated $\text{vis à vis}$ the kinetics of ATP hydrolysis to suggest allosteric modulation. Finally, as depicted in Fig. 4A, binding of the nonhydrolyzable nucleotide AMPFPNP does not stimulate Vi-induced trapping. If binding of nucleotide at one ATP site exerts a positive allosteric control over hydrolysis at the other, stimulation of hydrolysis should result in an increase in Vi-induced nucleotide trapping.

On the basis of this study, we propose a model for the catalytic cycle of Pgp, which is an extension of the one we proposed recently (10), and this is illustrated in Fig. 8. The drug and ATP first bind to Pgp (step I), because there is no energetic requirement for this step (36). Additionally, drugs do not affect nucleotide binding (Fig. 1) nor does nucleotide influence drug binding of substrate. The binding of nucleotide and drug is followed by the first hydrolysis event (step II), which is accompanied by a conformational change that reduces the affinity of both substrate (Ref. 10 and Fig. 2A) and nucleotide (Figs. 2C and 3A) for Pgp. This intermediate can be trapped by using Vi, an analogue of P, that generates the stable Pgp-ADP-Vi complex (step IIIA). Following hydrolysis, ADP is released (step IV). This release occurs spontaneously and is not influenced by the presence of...
nucleotides (Fig. 4B). The dissociation of ADP is accompanied by a conformational change that allows nucleotide binding (Fig. 5B) but substrate binding continues to be reduced (Fig. 6). A second ATP hydrolysis event is then initiated (step V) which is kinetically indistinguishable from the first (Fig. 7), at which point the substrate binding is still not regained (Fig. 6). This event too can be captured as an intermediate using Vi to trap the nucleotide (steps VIA and VIB). The subsequent release of ATP (step VII) completes one catalytic cycle bringing the Pgp molecule back to the original state where it can bind both substrate and nucleotide to initiate the next cycle (Fig. 6). This revised model thus incorporates key elements based on the work reported here to the scheme that we proposed earlier (10). The conformation of Pgp, following ATP hydrolysis, shows reduced affinity for the nucleotide (steps II, III, IIIA, V, VI, and VIA). Additionally, following the second ATP hydrolysis event, the release of ATP from Pgp is essential to complete the catalytic cycle, i.e. to bring the molecule back to the state where it will bind the next molecule of drug substrate (steps VIB and VII). Finally, this model is consistent with our recent finding that ADP release at steps IV and VII (16) appear to be the rate-limiting steps in the catalytic cycle.

The model we propose invokes two hydrolysis events (Fig. 8, steps II and V) during each catalytic cycle. We have also demonstrated here that the two hydrolysis events in a single catalytic cycle are kinetically identical (Figs. 5B and 7) and differ only with respect to the status of the molecule via a vis substrate binding (Fig. 6). This too is consistent with the notion that ATP does not show preferential affinity to either ATP site, and the sites are recruited randomly for hydrolysis (see above). The recent resolution of the crystal structure of the soluble ATP subunit, ArsA of the bacterial arsenic efflux pump (37) and ATP subunits of bacterial ABC transporters MutS (38, 39) and MalK (40) that exhibit structural and functional similarity to Pgp, shows that the two functional ATP sites, are each composed of residues from both the N- and the C-terminal ATP sites. Such a tertiary structural organization is plausible for Pgp where the nucleotide binding domains in the N- and C-terminal halves of the protein each contribute to both ATP binding and hydrolysis with similar kinetic properties. The resolution of the structure of the Pgp ATP domains and further work on site-directed mutagenesis of residues in ATP sites should provide additional insights into the mechanism of the catalytic cycle of ATP hydrolysis.

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