Characterization of an Asymmetric Occluded State of P-glycoprotein with Two Bound Nucleotides

IMPLICATIONS FOR CATALYSIS

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P-glycoprotein (ABCB1), a member of the ABC superfamily, functions as an ATP-driven multidrug efflux pump. The catalytic cycle of ABC proteins is believed to involve formation of a sandwich dimer in which two NBD molecules are bound at the surface of the nucleotide binding domains (NBDs). However, such dimers have only been observed in isolated NBD subunits and catalytically arrested mutants, and it is still not understood how ATP hydrolysis is coordinated between the two NBDs. We report for the first time the characterization of an asymmetric state of catalytically active native P-glycoprotein with two bound molecules of adenosine 5′-(γ-thio)triphosphate (ATPγS), one of low affinity ($K_d 0.74 \text{ mM}$), and one “occluded” nucleotide of 120-fold higher affinity ($K_d 6 \text{ μM}$). ATPγS also interacts with P-glycoprotein with high affinity as assessed by inhibition of ATP hydrolysis and protection from covalent labeling of a Walker A Cys residue, whereas non-hydrolyzable ATP analogues do not. Binding of ATPγS (but not ATP) causes Trp residue heterogeneity, as indicated by collisional quenching, suggesting that it may induce conformational asymmetry. Asymmetric ATPγS-bound P-glycoprotein does not display reduced binding affinity for drugs, implying that transport is not driven by ATP binding and likely takes place at a later stage of the catalytic cycle. We propose that this asymmetric state with two bound nucleotides represents the next intermediate on the pathway toward ATP hydrolysis after nucleotide binding, and that an alternating sites mode of action is achieved by simultaneous switching of the two active sites between high and low affinity states.

The ATP-binding cassette (ABC) protein superfamily is a large and diverse group of (primarily) active membrane transporters found in all organisms from bacteria to humans (1–3). Proteins in this superfamily share a similar domain organization, with two membrane-embedded transmembrane domains and two cytoplasmic nucleotide binding domains (NBDs). These four domains are often expressed as separate subunits in prokaryotic ABC proteins. In eukaryotes, ABC proteins consist of either single fused polypeptides comprising all four domains or “half-transporters” with a single transmembrane domain and NBD that operate as homo- or heterodimers. Binding and hydrolysis of ATP at the NBDs induce conformational changes that drive transport of substrate across the membrane. The NBDs of ABC proteins comprise three highly conserved sequences; the Walker A and B motifs, commonly found in nucleotide-binding proteins, and the Signature C motif, which is unique to this protein family (4).

P-Glycoprotein (Pgp, ABCB1), the best studied eukaryotic ABC transporter, is a 180-kDa plasma membrane protein that functions as an efflux pump for amphipathic natural products, drugs, and peptides (5, 6). Pgp has been implicated in the phenomenon of multidrug resistance, which is often observed in human cancers and represents a major impediment to the successful treatment of tumors using chemotherapy (7). Both NBDs of Pgp are capable of ATP binding and hydrolysis (8). Based on work showing trapping of ortho-vanadate in only one of the catalytic sites of Pgp (9), Senior et al. (10, 11) proposed an alternating sites mechanism for the transporter in which only one active site is able to hydrolyze ATP at any point in time, with the two sites taking turns at catalysis. An alternating sites mechanism requires that all reaction intermediates are asymmetric, thus providing “memory” of which active site last turned over. The mechanism of Pgp-mediated drug transport has been the focus of intensive study (12, 13), but how ATP hydrolysis during the catalytic cycle is coordinated between the two NBDs at the molecular level and how this is coupled to drug transport are still not understood.

An emerging consensus in the ABC protein field over the past few years has been that dimerization of the NBDs, which is driven by nucleotide binding, appears to be an essential step in the transport cycle. High resolution x-ray crystal structures of isolated bacterial NBD subunits and entire bacterial ABC proteins have revealed interdigitated “head-to-tail” dimers, where two molecules of ATP are bound at the dimer interface by the Walker A and B motifs of one NBD and the C motif of the opposing NBD (see for example, see Refs. 14–18), an arrangement that was previously predicted (19). However, such stable nucleotide sandwich dimers have been found only when ATP hydrolysis is blocked either by the absence of Mg$^{2+}$ or by mutation of an essential catalytic residue, and they have not yet been
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observed in a catalytically active protein. Biochemical studies and simulations on bacterial ABC proteins have demonstrated that binding of ATP, but not ADP, induces dimerization of the NBDs (20–24). It is now clear that an ABC protein conformation with two bound nucleotides is required to initiate the catalytic cycle; however, the symmetrical nature of the crystallographic sandwich dimers suggests that they probably do not represent a true catalytic intermediate (25). The recent x-ray crystal structure of Pgp with bound peptide substrate molecules was determined in the absence of nucleotide (26). Although the two NBDs appear to be located close to one another, they do not appear to be tightly associated, and thus, this structure does not provide any additional information on their mode of interaction during catalysis.

Combined mutation of the two “catalytic carboxylates” (Glu-556/1201; human Pgp) in the NBD Walker B motifs of Pgp resulted in a protein that displayed tight binding of 8-azido-ATP (27). Tombline et al. (28, 29) were the first to report the isolation of an occluded state with ATP tightly bound at a maximal 1:1 stoichiometry in the catalytically inactive Pgp double-mutant (E552A/E1197A; mouse Pgp). The enzyme appears to be arrested in an occluded nucleotide conformation similar to that of a stabilized NBD dimer, representing a (normally) transient, asymmetric, catalytic intermediate (25). More recently, it was reported that a single molecule of the non-hydrolysable ATP analogue ATPγS was occluded by wild-type Pgp (30), suggesting that this resembles the E-S intermediate in the catalytic cycle of the native transporter.

We seek to understand how NBD dimerization drives ATP hydrolysis by Pgp and determine the biochemical nature of the E-S intermediate that immediately precedes the ATP hydrolysis step. In the present work we report the biochemical and spectroscopic characterization of an asymmetric state of wild-type catalytically active Pgp with two bound molecules of ATPγS. One nucleotide is bound with low affinity, and the other is an occluded nucleotide that binds with 120-fold higher affinity. Binding of two other non-hydrolysable ATP analogues did not induce an asymmetric state. ATPγS was also found to interact with Pgp with high affinity as assessed by inhibition of ATP hydrolysis and protection from covalent labeling of Cys residues in the NBDs. Binding of ATPγS (but not ATP) was found to induce Trp residue heterogeneity, as indicated by collisional quenching, suggesting the existence of conformational asymmetry in the Pgp molecule. This asymmetric occluded state containing two bound nucleotides still displays high affinity binding of several drug substrates and modulators, which has important implications for the catalytic and transport cycle of Pgp. We propose a site-switching mechanism that leads to alternation of catalysis between the two active sites.

EXPERIMENTAL PROCEDURES

Materials—Acrylamide was obtained from Bio-Rad. KI was purchased from Fisher. Disodium-ATP, disodium-ADP, AMP-PCP, AMP-PNP, CHAPS, N-acetyl-L-tryptophanamide, sodium ortho-vanadate, sodium thiophosphate, vinblastine, daunorubicin, and verapamil were purchased from Sigma. ATPγS tetralithium salt was obtained from Biolog Life Science Institute (Bremen, Germany). Propafenone GP12 (31) was a gift from Dr. Peter Chiba (Institute of Medical Chemistry, Medical University of Vienna). e-ATP and 2-(4’-maleimidylalanino)naphthalene-6-sulfonic acid (MIANS) were supplied by Invitrogen. 8-Azido-ATP was purchased from Affinity Labeling Technologies (Lexington, KY). ATPγS[^35]S (specific activity 65 Ci/mmol) was supplied by PerkinElmer Life Sciences.

Pgp Purification—Plasma membrane vesicles were isolated from multidrug-resistant CH^4^B30 Chinese hamster ovary cells and stored at −80 °C. Pgp was purified from CH^4^B30 plasma membrane as described previously (32) by initial extraction with 25 mM CHAPS buffer followed by solubilization of the S1 pellet in 15 mM CHAPS buffer. The protein was further purified by affinity chromatography on a concanavalin A-Sepharose column. The final product consisted of 90–95% pure Pgp in 2 mM CHAPS, 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MgCl_2, pH 7.5. The purified Pgp preparation was kept on ice and used within 24 h. Plasma membrane protein was quantitated by the method of Bradford (33), and a modified Lowry assay was used to determine the concentration of purified Pgp (34) using bovine serum albumin (crystallized and lyophilized, Sigma) as a standard. Highly stable vanadate-trapped Pgp was prepared using Co^2+ ions as previously described (35, 36).

Inhibition of Pgp ATPase Activity by Nucleotides and Nucleotide Analogues—The Mg^2+ -ATPase activity of Pgp was determined by measuring the release of inorganic phosphate from ATP. Purified Pgp was incubated with assay buffer (50 mM Tris-HCl, 5 mM MgCl_2, pH 7.5) in the presence of 2 mM ATP at 37 °C for 20 min as described earlier (37). To determine IC_{50} values for the inhibition of ATPase activity as a function of concentration and incubation time, purified Pgp was preincubated for 5 min with increasing concentrations of ATPγS or other ATP analogues, ATP was added, and activity was determined as described above. Details for each experiment are given in the figure legends. To determine the K_i values for the inhibition of Pgp, ATPase activity was measured as a function of ATP concentration for several different nucleotide analogue concentrations. For classical competitive inhibition, the K_i value was estimated by fitting the rate of ATP hydrolysis at increasing ATP concentrations to kinetic equations (using SigmaPlot, Systat Software, Chicago, IL). For noncompetitive inhibition, IC_{50} values were estimated instead.

Protection of Pgp ATPase Activity from MIANS Inactivation by Nucleotides and Nucleotide Analogues—Covalent labeling of Pgp at Cys-428 and Cys-1071 within the NBD Walker A motifs was achieved using the thiol-specific probe, MIANS, as described previously (32). After incubation of Pgp with increasing concentrations of nucleotide or nucleotide analogue for 5 min, 20 μM MIANS was added (from a 2.5 mM stock solution in methanol), and the protein was further incubated for 10 min followed by the addition of 2 mM dithiothreitol to quench the reaction. After 2 min, 2 mM ATP was added, and the ATPase activity was determined as described above. No MIANS was added to the control samples. IC_{50} values for the protection of Pgp ATPase activity from MIANS inactivation were estimated from the difference in ATPase activity between Pgp incubated with and without MIANS as a function of nucleotide concentration.
Hydrolysis of ATP\(^{35}\)S by Pgp—Purified Pgp in assay buffer was incubated with 0.1 mM ATP\(^{35}\)S and 1 \(\mu\)Ci of ATP\(^{35}\)S (50 \(\mu\)l total volume) for times ranging from 0 to 4 h at 37 °C. A 5-\(\mu\)l aliquot of each sample was spotted onto activated silica gel 60 F254 aluminum-backed TLC sheets (Merck) and separated as described (38). The radioactivity corresponding to ATP\(^{35}\)S and \(^{35}\)S-labeled triphosphate was quantitated using a storage phosphor screen with a Bio-Rad Personal Molecular Imager. Purified Pgp was incubated with unlabeled ATP under identical conditions for 0–4 h, the products were separated by TLC, and the extent of hydrolysis was determined by imaging under UV illumination.

**Stoichiometry of ATP\(^{35}\)S Binding to Pgp Using Equilibrium Dialysis**—Equilibrium dialysis measurements were performed using several 250-\(\mu\)l Micro-Equilibrium Dialyzer 2-Chamber Systems, using membranes with a molecular mass cutoff of 100 kDa between the two chambers (Harvard Apparatus, St. Laurent, QC, Canada). All the samples were prepared in 2 mM CHAPS, 2 mM dithioerythritol, 20 mM HEPES, 100 mM NaCl, 5 mM MgCl\(_2\) buffer, pH 7.4. For binding experiments, 125 \(\mu\)l of purified Pgp (0.3 mg/ml in 2 mM CHAPS buffer) was placed in one chamber of the dialyzer with increasing concentrations of ATP\(^{35}\)S (ranging from 10 to 100 \(\mu\)M, in the same buffer) in the opposite chamber. In parallel, 125 \(\mu\)l of buffer alone and the same increasing concentrations of ATP\(^{35}\)S were employed in separate dialyzers. The dialyzers were placed on a shaker for ~18 h at 4 °C. After this equilibration period, 5-\(\mu\)l aliquots of sample (in triplicate) were used to determine the amount of protein in each chamber (based on a molecular mass of 140 kDa). 30-\(\mu\)l aliquots of each chamber sample (in triplicate) were added to 10 ml of BCS scintillant (Amersham Bio- sciences) for counting in a Beckman LS 6000SE scintillation counter. A standard curve was generated for ATP\(^{35}\)S by counting several dilutions of the stock solution and used to calculate the total pmol of ATP\(^{35}\)S in each protein chamber and the pmol of free ATP\(^{35}\)S in each buffer chamber. The bound ATP\(^{35}\)S was calculated as the difference between the total ATP\(^{35}\)S and the free ATP\(^{35}\)S. The molar ratio of bound ATP\(^{35}\)S to Pgp was used to calculate the binding stoichiometry. The ATPase activity of Pgp was determined before and after dialysis, and the proportion was shown to retain >95% of its catalytic function after dialysis.

**Fluorescence Quenching Studies**—A PTI Alphascan-2 spectrofluorimeter (Photon Technology International, London, ON, Canada) was used to carry out fluorescence measurements at 22 °C using a quartz microcuvette (Hellma, Germany). Fluorescence quenching experiments with nucleotides and drugs were carried out using 250 \(\mu\)l of Pgp solution (50 \(\mu\)g/ml in 2 mM CHAPS buffer, pH 7.5) at 22 °C. The working solutions for titrations were prepared in water for nucleotides and in DMSO for drugs. For quenching of Pgp Trp fluorescence and Pgp-MIANS fluorescence, excitation/emission was at 290/320 and 320/420 nm, respectively, with 4-nm slits. Native Pgp and Pgp-MIANS samples were titrated with either nucleotides or drugs, and after each addition the samples were equilibrated for 40 s. The measured fluorescence emission intensities were corrected for dilution, scattering, and the inner filter effect as described earlier (32). Changes in fluorescence intensity were routinely expressed as the percent change induced by substrate binding relative to the absolute intensity measured in the absence of substrate. Experimental data showing monophasic or biphasic quenching behavior were fitted to the equation (39)

\[
\frac{\Delta F}{F_0} \times 100 = \frac{(\Delta F_{\text{max}1} F_0 \times 100) \times [S]}{K_{d1} + [S]} + \frac{(\Delta F_{\text{max}2} F_0 \times 100) \times [S]}{K_{d2} + [S]} \quad (\text{Eq. 1})
\]

where \(F_0\) is the initial fluorescence intensity, \(\Delta F/F_0 \times 100\) is the percent quenching (percent change in fluorescence intensity relative to the initial value), \([S]\) is the substrate concentration, and \(K_{d1}\) and \(K_{d2}\) are the dissociation constants. Fitting to a one-site or two-site model was carried out by nonlinear regression using SigmaPlot, and values for \(K_Q\) and the maximum percent fluorescence quenching \(\Delta F_{\text{max}Q}/F_0 \times 100\) were extracted.

**Collisional Quenching Studies**—Stock solutions of 5 m acrylamide, 5 m KI, and 5 m CsCl were added as 2.5-\(\mu\)l aliquots in buffer to 250 \(\mu\)l of 50 \(\mu\)g/ml Pgp in 2 mM CHAPS buffer. All quencher solutions were freshly prepared, and 0.1 mM Na\(_2\)S\(_2\)O\(_3\) was added to the KI stock solution to prevent I\(_3\)\(^-\) formation. Fluorescence emission was measured at 320 nm after excitation at 290 nm, and fluorescence intensities were corrected for dilution and scattering. In control titrations, KCl was added at the same concentrations as KI to correct for any ionic strength effects. Parallel experiments were carried out using N-acetyl-L-tryptophanamide to assess fluorescence quenching by the same agents when Trp is completely accessible in aqueous solution. Quenching data for a homogeneous single fluorophore system were analyzed using the Stern-Volmer equation (40),

\[
F_o/F = 1 + K_{SV}[Q] \quad (\text{Eq. 2})
\]

where \(F_o\) and \(F\) are the fluorescence intensities in the absence and presence of quencher, respectively, \([Q]\) is the concentration of quencher agent, and \(K_{SV}\) is the Stern-Volmer quenching constant. For complex quenching including at least two types of fluorophores (41), the following equation was used,

\[
F_o/F = \left( \frac{\sum_{i=1}^{n} f_i}{(1 + K_{SV}[Q])} \right)^{-1} \quad (\text{Eq. 3})
\]

where \(f_i\) is the fractional contribution of component \(i\) to the total fluorescence \(F\).

**RESULTS**

**Affinity of Pgp for Binding Nucleotides and Nucleotide Analogues**—Pgp from hamster and mouse contains 11 Trp residues, 8 of which are located in regions of the protein close to the membrane-water interface (42), according to the recent crystal structure (26). We previously showed that the Trp fluorescence emission maximum of Pgp is indicative of a relatively hydrophobic environment (43). The Trp residues that contribute to emission are sensitive to binding of both drugs and nucleotides, and this can be used to quantify the affinity of their interactions by fluorescence quenching titrations (43). This approach has the advantage of using native, nonmodified, catalytically active Pgp. The affinity of Pgp was deter-

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Figure 1. A, quenching of the intrinsic Trp fluorescence of Pgp by binding of nucleotides and nucleotide analogues at 22 °C is shown. Increasing concentrations of AMP-PNP (A), AMP-PCP (B), and ATPS (C) were added to purified Pgp in buffer containing 2 mM CHAPS. D, ATPS was added to vanadate-trapped Pgp in buffer containing 2 mM CHAPS. Fluorescence emission at 320 nm was recorded after excitation at 290 nm. The data points represent the means ± S.E. for triplicate determinations. Where error bars are not visible, they are contained within the symbols. The inset in Fig. 1C shows the high affinity binding (occlusion) of ATPS in the concentration range 0 – 35 mM.

Table 1

| Nucleotide | $K_d$ (mM) | $\Delta F_{max}/F_0 \times 100$ |
|------------|------------|-------------------------------|
| ATP        | 0.87 ± 0.12 | 8.23 ± 0.54 |
| ADP        | 0.33       | 9.46 |
| AMP-PNP    | 0.39 ± 0.06 | 8.11 ± 0.46 |
| AMP-PCP    | 0.16 ± 0.01 | 11.9 ± 0.15 |
| ATPS       | 0.0062 ± 0.0004 ($K_d$) | 3.98 ± 0.72 |
| 8-Azido-ATP| 0.74 ± 0.42 ($K_d$) | 8.02 ± 1.42 |
| e-ATP      | 0.0132 ± 0.0004 ($K_d$) | 4.16 ± 0.59 |

* Determined by Trp quenching for all nucleotides except 8-azido-ATP, which was determined by quenching of Pgp-MIANS. Values represent the means of triplicate determinations.

* Binding was carried out at 22 °C for all nucleotides except ATP, where experiments were performed at 10 °C to limit hydrolysis by Pgp. $K_d$ for binding of ATP at 22 °C was previously reported to be 0.28 mM (43).

* Values reported previously (43).

* Data were fitted to an equation for binding to two sites with affinities $K_{d1}$ and $K_{d2}$ (see Experimental Procedures).

* Binding to the vanadate-trapped state of Pgp.

* Not determined; the high intrinsic fluorescence of this compound interferes with quenching assays.

mined for the non-hydrolyzable analogues AMP-PNP, AMP-PCP, and ATPS and the photoaffinity label 8-azido-ATP. ATP and ADP were also included for comparison; the binding affinity of these nucleotides was determined previously using fluorescence quenching approaches (32, 43, 44). Titration of Pgp with increasing concentrations of nucleotide analogues led to saturable, concentration-dependent quenching, which fit to a hyperbolic binding curve for AMP-PNP (Fig. 1A), AMP-PCP (Fig. 1B), and ATP (binding was determined at 10 °C to prevent hydrolysis). Because 8-azido-ATP undergoes photolytic reaction on irradiation at 290 nm, it was not possible to estimate its binding affinity using Trp quenching. Therefore, Pgp was labeled on two Cys residues within the NBDs using the fluorophore MIANS, and the binding affinity for 8-azido-ATP was estimated by quenching of Pgp-MIANS fluorescence, as described earlier (32). Assuming an interaction with a single type of binding site, the quenching data were used to estimate the $K_d$ for binding of the nucleotide analogues (Table 1). All nucleotides displayed low affinity binding, with AMP-PNP showing comparable affinity to ATP, whereas AMP-PCP and 8-azido-ATP displayed slightly higher affinity.

The ATPS Trp quenching curve was characterized by more complex biphasic behavior, indicating the presence of two binding sites of differing affinity (Fig. 1C). Fitting of the data to a two-site equation revealed the existence of two binding sites for ATPS with remarkably different binding affinities. Indeed, one site binds ATPS with very high affinity ($K_d$ of 6 μM; Fig. 1C, inset) and the second binds ATPS with much lower affinity ($K_d$ of 0.74 mM). The low binding affinity is comparable with that for unmodified ATP, and the high binding affinity exceeds that by ~120-fold (Table 1). Similar high binding affinity for ATP was reported in the occluded state of the catalytically arrested E552A/E1197A mutant Pgp (25, 28, 29), but this is the first report of two nucleotide binding sites of such widely differing affinity in wild-type catalytically active Pgp. As proposed by Senior and co-workers (25, 29), the tightly bound occluded nucleotide molecule is likely committed to hydrolysis, although in this case it is not readily hydrolyzable (see below).

Pgp-mediated Hydrolysis of Nucleotides and Nucleotide Analogues—Although ATPS is described as a non-hydrolyzable ATP analogue, there have been reports that it can be hydrolyzed at a low rate by some transport ATPases (see for example Refs. 45 and 46). Pgp expressed in insect cells appeared to be unable to hydrolyze this analogue (30). Using a high sensitivity radiometric assay, we observed that Pgp-mediated hydrolysis of ATPS$[^{35}]$S to $[^{35}]$Sphosphate was linear with time over a period of 0 – 4 h, with ~25% hydrolyzed after a 3-h incubation with 10 μg of Pgp under the assay conditions. The rate of hydrolysis of ATPS was ~0.4% that determined for ATP under the same conditions. Interestingly, this is compara-
able to the rate reported for the E552A/E1197A mutant Pgp, which forms the occluded state in the presence of ATP (28). For 8-azido-ATP, the $K_m$ for hydrolysis was substantially lower than that of unmodified ATP and correlated well with the $K_d$ value for binding to Pgp. The relative $V_{max}$ (compared with ATP) for Pgp-mediated hydrolysis was reduced to 30% for 8-azido-ATP. Previously, a $K_m$ value of 0.4–0.6 mM and an 8–10-fold reduced hydrolysis rate were reported for 8-azido-ATP (27). The $K_m$ and relative $V_{max}$ values were also determined for hydrolysis of $\epsilon$-ATP (Table 2); however, it was not possible to determine the binding affinity of this analogue, as its intrinsic fluorescence interferes with the quenching assays and is not environmentally sensitive. In general, the high $V_{max}$ values of 8-azido-ATP and $\epsilon$-ATP limit the usefulness of these analogues, as they are rapidly hydrolized by Pgp.

### Inhibition of Pgp-mediated ATP Hydrolysis by Nucleotides and Nucleotide Analogues

The basal ATPase activity of Pgp can be inhibited by nucleotides and nucleotide analogues that compete with ATP for interaction at the NBDs. To distinguish between competitive and noncompetitive inhibition, the rate of ATP hydrolysis was followed as a function of ATP concentration in the presence of several fixed concentrations of various nucleotide inhibitors. AMP-PNP displayed competitive inhibition (Fig. 2A), with characteristic double reciprocal plots (Fig. 2A, inset) that led to an estimated $K_i$ value of 0.53 mM, the same as the $K_m$ for ATP hydrolysis (Table 2) and comparable with the estimated $K_d$ for binding (Table 1). ADP was also previously shown to inhibit ATP hydrolysis competitively (37). There has been a report that AMP-PCP did not inhibit Pgp-mediated ATP hydrolysis, even at concentrations as high as 10 mM (30). However, in our experimental system, this nucleotide analogue inhibited ATP hydrolysis with the inhibition plot exhibiting sigmoidal character (Fig. 2B). Fitting to the Hill equation ($n = 1.77$) gave a $K_i$ value of 1.6 mM for AMP-PCP (Table 2), which is about 10-fold higher than its $K_m$ for binding Pgp. ATPγS inhibited ATP hydrolysis at very low concentrations (Fig. 2C), with an estimated IC$_{50}$ value of 6 μM (Table 2), which agrees well with the high affinity $K_d$ value found for this nucleotide analogue (Table 1). The IC$_{50}$ values for inhibition of ATP hydrolysis by 8-azido-ATP and $\epsilon$-ATP could not be determined as these analogues are rapidly hydrolized by Pgp.

### Protection of Pgp from MIANS Inactivation by Nucleotides and Nucleotide Analogues

The fluorescent compound MIANS inactivates Pgp by a covalent reaction with Cys-413 and Cys-1074 located within the Walker A motifs of the NBDs (32, 47). Although these residues are not necessary for Pgp function, they are located close to the catalytic sites, and their modifica-
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tion with the relatively large MIANS group leads to complete loss of ATPase activity (32). However, MIANS-labeled Pgp displays binding of both nucleotides and drugs with normal affinity. ATP can protect Pgp from MIANS inactivation in a concentration-dependent manner (32), with an IC50 value of ~0.3 mM (Table 2), consistent with its Kd for binding. Thus, IC50 values for protection from MIANS inactivation by various nucleotides and nucleotide analogues were determined as an additional measure of their interaction with the NBDs of Pgp (Table 2). The IC50 for protection by AMP-PNP was similar to the Kd for binding (Fig. 3A) and the Kd for inhibition of ATPase activity; thus, this nucleotide analogue behaves as predicted. In contrast, AMP-PCP protected Pgp from MIANS inactivation with an IC50 similar to that for inhibition of ATP hydrolysis (Fig. 3B) but ~12-fold higher than its Kd for binding to Pgp. Thus, AMP-PCP displayed anomalous behavior. This unusual pattern suggests that AMP-PCP is not a good choice as a non-hydrolyzable nucleotide analogue for use with Pgp. ATPγS protected Pgp from MIANS inactivation at very low concentrations (Fig. 3C, IC50 of 5 μM), consistent with its IC50 for inhibition of ATP hydrolysis and the high affinity component of its binding.

Stoichiometry and Characterization of High Affinity ATPγS Binding to Pgp (Occlusion)—It was previously reported that one molecule of ATP or 8-azido-ATP is tightly bound (occluded) in the catalytically arrested Walker B E556/1201Q double mutant of Pgp (28, 48). We used equilibrium dialysis to measure the stoichiometry of ATPγS binding to wild-type Pgp at low concentrations where only the high affinity binding site is significantly occupied. As shown in Fig. 4, results showed that a single molecule of ATPγS is occluded in the NBD under these conditions. Sauna et al. (30) previously reported that ATPγS could not be occluded at 4 °C; however, our equilibrium dialysis experiments were carried out at this temperature, and occlusion clearly does take place. However, there are differences in the ATPγS concentration dependence of the equilibrium dialysis data (Fig. 4) and the fluorescence binding assays (Fig. 1C; carried out at 22 °C), which likely arise from the different temperatures at which these experiments were conducted. Tomblin et al. (28) also found that nucleotide occlusion was a highly temperature-dependent process. Sauna et al. (30) reported that ATPγS is not occluded in the presence of a 10-fold higher concentration of ATP and attributed this to blocking of the high affinity site by ATP. In our case, this scenario seems unlikely, as the binding affinity of the occluded nucleotide is 120-fold higher than that of the more loosely bound molecule. We found that a 20-fold excess of ATP, as expected, had only a small effect on the ability of ATPγS to bind to Pgp, as assessed by Trp quenching and the IC50 value for inhibition of ATP hydrolysis (data not shown).

Interaction of ATPγS with Vanadate-trapped Pgp—In the presence of vanadate, Pgp forms a stable complex in which ADP and ortho-vanadate are trapped in only one catalytic site after a single round of ATP hydrolysis (9). This trapped state is believed to be a structural mimic of the catalytic transition state and is thought to represent the conformation formed immediately “post-hydrolysis.” We previously showed that the unoccupied NBD of vanadate-trapped Pgp is able to bind fluorescent ATP and ADP analogs, and vanadate-trapped Pgp-MIANS can bind ATP and ADP (35, 36). When the stable vanadate-trapped Pgp complex formed in the presence of Co2+ ions (35, 36) was evaluated for binding of ATPγS using Trp quenching, only a single hyperbolic curve was observed with a Kd value of 13.2 μM (Fig. 1D and Table 1). This binding affinity resembles that of the tightly bound occluded ATPγS molecule found in basal-state Pgp.
Effect of ATPγS Occlusion on Drug Binding to Pgp—To determine the effect of ATPγS occlusion on the ability of Pgp to interact with its drug substrates, we used Trp quenching to measure the binding affinity for two multidrug resistance drugs

![Graph](image)

**FIGURE 4.** Stoichiometry of high affinity binding of ATPγS to Pgp (occlusion). Purified Pgp in 2 mM CHAPS buffer was loaded into dialyzers containing 100 kDa molecular mass cut-off filters and equilibrated with various concentrations of ATPγS for 18 h at 4 °C. The molar ratio of occluded ATPγS to Pgp in each sample was estimated as described under “Experimental Procedures” by liquid scintillation counting of the radioactivity and determination of the protein concentration in each chamber. Data points represent the means ± S.E. of triplicate determinations. Where error bars are not visible, they are contained within the symbols.

![Graph](image)

**FIGURE 5.** Drug binding affinity of Pgp in the presence of ATP and ATPγS. Purified Pgp in CHAPS buffer was preloaded with nucleotide at 22 °C. Increasing concentrations of drugs were titrated with Pgp at 22 °C, and the Trp fluorescence emission at 320 nm was recorded after excitation at 290 nm. ○, no nucleotide; ▲, 4 mM ATP; ▼, 0.2 mM ATPγS. The percent quenching of fluorescence (∆F/Fo × 100) was calculated relative to the fluorescence of Pgp in the absence of drugs. Data points represent the means ± S.E. for triplicate determinations. The continuous line represents the best computer-generated fit of the data points (shown by the symbols) to an equation describing interaction of drug with a single type of binding site (see “Experimental Procedures”).

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![Graph](image)

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(vinblastine and daunorubicin) and two modulators (verapamil and propafenone GP12) in the absence and presence of ATP and ATPγS (Fig. 5). The data show that for all four drugs, the dissociation constants are similar in the absence of nucleotide and with bound ATP and ATPγS (Table 3). The K_{sv} values for ATPγS were also estimated in the presence of vinblastine and verapamil at a concentration of 50 μM, and no significant change in the nucleotide binding affinities was observed (data not shown).

Collisional Fluorescence Quenching Studies of Pgp with Occluded ATPγS—Collisional quenching has proved to be a very useful technique to study the aqueous accessibility of fluorescent groups within Pgp and also to provide information on the polarity and charge of the region in the vicinity of the fluorophore (43). Changes in accessibility after ligand binding can be indicative of a change in protein conformation. To investigate conformational differences in more detail, Pgp in the presence of saturating concentrations of either ATP or ATPγS was titrated with increasing concentrations of three collisional quenchers, acrylamide, I^{-} ion, and Cs^{+} ion. In each case, a parallel quenching experiment was carried out with N-acetyl-L-tryptophanamide, a soluble Trp analogue.

As we showed earlier (43), the neutral acrylamide molecule was a poor quencher of Pgp Trp residues, with a 13-fold lower Stern-Volmer quenching constant compared with N-acetyl-L-tryptophanamide (Table 4). The completely linear Stern-Volmer plot suggests that only a single class of Trp residues within Pgp is quenched, and they all are equally accessible to acrylamide (Fig. 6A). No significant difference was observed for acrylamide quenching of Trp residues when Pgp was preincubated with saturating levels of either ATP or ATPγS (Fig. 6A); indeed, the Stern-Volmer constants, K_{sv} values, are very similar (Table 4). Thus, the accessibility of Trp residues to acrylamide is unchanged in the absence and presence of ATP and ATPγS. However, a different situation was observed for collisional quenching of Pgp by I^{-}. In the ATP-bound state we observed a linear Stern-Volmer plot, again indicating the existence of a single class of Trp fluorophore. In contrast, for the ATPγS-bound state (both high and low affinity sites occupied), the Stern-Volmer plot was curved, indicating the existence of at least two classes of fluorophore with different accessibilities to quencher (40, 41). The first group of Trp residues is highly inaccessible to I^{-} quenching as indicated by the K_{sv} value, which is 3.5-fold lower than that of nucleotide-
free Pgp (Table 4) and represents ~77% of the total fluorescence intensity. The second group of Trp residues represents ~23% of the total fluorescence intensity and is quenched much more efficiently, with a $K_q$ value 5.6-fold higher than that of nucleotide-free Pgp (Table 4). The 77/23 distribution of Trp residues between these two groups cannot be readily translated into absolute numbers of residues, as not all of the 11 Trp residues found in Pgp contribute to the overall fluorescence emission (42). Overall, these results indicate that some Trp residues are more shielded from $\Gamma^-$ when ATPγS binds, whereas others are more accessible, suggesting that the nucleotide induces heterogeneity in Trp residue accessibility that is not seen when ATP binds. One explanation is that ATPγS binding may induce an asymmetrical protein conformation. We also conducted similar experiments with the positive collisional quencher Cs$^+$, but the level of Pgp quenching was very low (data not shown). Taken together, the results obtained with charged quenchers also suggest that the protein regions around the emitting Trp residues are positively charged, as Cs$^+$ ions cannot closely approach them, whereas $\Gamma^-$ ions can.

**DISCUSSION**

ATP-driven dimerization of the NBDs is recognized as playing a key role in the catalytic cycle of ABC proteins; however, the details of how this is linked to ATP hydrolysis and substrate transport remain unclear. The alternating sites model for the catalytic mechanism of ATP hydrolysis by Pgp is supported by the observation of vanadate trapping in only one catalytic site and the isolation of an occluded state of the E552A/E1197A double mutant containing a single tightly bound nucleotide. More recently, it has become clear that ATPγS also induces the occluded state in wild-type Pgp, and thus, this non-hydrolyzable analogue may be an invaluable tool to dissect the steps in the catalytic pathway. We have investigated the interactions of ATPγS and other non-hydrolyzable ATP analogues with native, catalytically active Pgp using biochemical and fluorescence approaches. We obtained several quantitative parameters for nucleotide binding and hydrolysis, which led to a much more complete picture of the type of interactions of each species with the NBDs of Pgp, and enabled us to make some important conclusions about the mechanism of ATP hydrolysis and drug transport.

ATPγS was hydrolyzed at a low, but measurable rate by Pgp (~0.4% that observed for ATP). We cannot rule out that ATPγS hydrolysis is carried out by an impurity in the Pgp preparation, but it seems unlikely. Other membrane-bound ATPases such as

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**TABLE 3**

Effect of ATP and ATPγS on the drug binding affinity of Pgp

| Drug            | Bound nucleotide | $K_a$ | $\Delta F_{max}/F_0 \times 100^a$ |
|-----------------|------------------|-------|----------------------------------|
|                 |                  | µM   |                                   |
| Vinblastine     | ATP              | 0.73 ± 0.06 | 18.5 ± 0.3               |
|                 | ATPγS            | 1.36 ± 0.11 | 23.5 ± 0.4               |
| Daunorubicin    | ATP              | 11.0 ± 1.4 | 63.7 ± 2.7               |
|                 | ATPγS            | 10.0 ± 0.76 | 60.1 ± 1.5               |
| Verapamil       | ATP              | 9.11 ± 0.96 | 56.7 ± 1.8               |
|                 | ATPγS            | 3.05 ± 0.01 | 20.5 ± 1.4               |
| Propafenone GP12 | ATP             | 2.97 ± 0.54 | 34.4 ± 2.1               |
|                 | ATPγS            | 1.88 ± 0.49 | 17.9 ± 4.0               |

$^a$Estimates of the dissociation constant, $K_a$, and percent maximal quenching ($\Delta F_{max}/F_0 \times 100$) were obtained by fitting the fluorescence quenching data to an equation for a single binding site (the first term in Equation 1). Values represent the means for triplicate determinations.

**TABLE 4**

Stern-Volmer quenching constants for collisional quenching of Pgp bound to ATP and ATPγS

| Quencher | Stern-Volmer quenching constant, $K_{SV}$ |
|----------|------------------------------------------|
| NATA     | Acrylamide 20.1 ± 0.63 1.59 ± 0.02 1.58 ± 0.02 1.61 ± 0.02 |
| Pgp      | Acrylamide 4.44 ± 0.06 0.74 ± 0.02 0.72 ± 0.005 4.14 ± 2.7 ($S$) 0.21 ± 0.17 ($S$) |
| Pgp + ATP| Acrylamide 0.19 ± 0.24 0.05 ± 0.02 0.07 ± 0.001 0.96 ± 0.02 |
| Pgp + ATPγS| Acrylamide 0.01 ± 0.22 0.03 ± 0.01 0.05 ± 0.003 1.03 ± 0.02 |

$^b$The Stern-Volmer quenching constant for a homogeneous population of Trp residues was determined from the slope of the plot of $F_0/F$ versus quencher concentration (see Fig. 6, expressed as values ± fitting error.

$^c$NATA or Pgp samples in 2 mM CHAPS buffer in the absence or presence of ATP or ATPγS were titrated at 22 °C with increasing concentrations of acrylamide or KI solutions.

$^d$Parameters for two different populations of Trp residues were determined by fitting the quenching data (see Fig. 6) to Equation 3 (see “Experimental Procedures”), expressed as values ± fitting error.
the Na\(^{+}\)K\(^{+}\) and Ca\(^{2+}\)-ATPases hydrolyze ATP\(\gamma\)S at very low rates compared with ATP, 0.09\% (45) and 0.5\% (46), respectively. It seems unlikely that a low abundance contaminant could produce the level of hydrolysis that we observe. Such an ATPase would also have to be almost completely vanadate-inhibitable, as inhibition of the ATPase activity of purified Pgp by vanadate approaches 100\% (36).

Two molecules of ATP\(\gamma\)S were bound to the protein, one with low affinity and the other with 120-fold higher affinity (\(K_d = 6 \mu M\)), corresponding to an occluded nucleotide. The occluded state reported by Tombline et al. (28) with a single tightly bound ATP molecule (\(K_d = 3 \mu M\)) probably also contains a second loosely bound nucleotide that was lost during chromatographic separation. This asymmetric intermediate likely exists when native Pgp hydrolyzes ATP, but it is short-lived, as the tightly bound nucleotide is committed to hydrolysis and proceeds rapidly to the transition state. Previously, Sauna et al. (30) reported that Pgp had low binding affinity for ATP\(\gamma\)S (0.29 mM), comparable with that of ATP. However, they used an indirect method (inhibition of photoaffinity labeling) to measure binding, which failed to detect the high affinity site.

We found that the IC\(_{50}\) for inhibition of Pgp-mediated ATP hydrolysis by ATP\(\gamma\)S was also very low, 6 \(\mu M\), comparable with the \(K_d\) determined for its high affinity binding. This likely reflects that fact that occupation of only one NBD by ATP\(\gamma\)S is needed to arrest catalytic activity, as the two NBDs are tightly coupled. A low IC\(_{50}\) value for inhibition of ATP hydrolysis by ATP\(\gamma\)S was reported in 1994 by Senior and co-workers (49), who were among the first to study the ATPase activity of Chinese hamster Pgp in detail. Interestingly, in their experiments ATP\(\gamma\)S stands out among all tested ATP analogues, showing at least a 10-fold higher IC\(_{50}\) value compared with other nucleotides. More recently, an IC\(_{50}\) value of 31 \(\mu M\) was noted by Martin et al. (50). In contrast, Sauna et al. (30) reported an IC\(_{50}\) value for ATP\(\gamma\)S of 0.19 mM, which represents a low affinity interaction, comparable with the \(K_d\) for binding of ATP and ADP and the \(K_m\) for ATP hydrolysis. The reason for this discrepancy is not clear.

We also demonstrated that ATP\(\gamma\)S inhibited the inactivation of Pgp by MIANS with a very low IC\(_{50}\) value, whereas other ATP analogues required a >50-fold higher concentration to do so. Like MIANS, N-ethylmaleimide and 7-chloro-4-nitro-2-oxa-1,3-diazole inhibit ATP hydrolysis by reacting covalently within the catalytic sites of Pgp (51, 52). They also impair tight binding of ATP to the E552A/E1197A double mutant (29), suggesting that they and MIANS prevent formation of the occluded nucleotide conformation. Because reaction with MIANS “competes” with binding of ATP\(\gamma\)S, the IC\(_{50}\) value for blocking of MIANS inactivation will reflect the high affinity binding of ATP\(\gamma\)S that is required to lock Pgp in the occluded state. Thus, three different indicators of ATP\(\gamma\)S binding affinity are in the low \(\mu M\) concentration range.

AMP-PNP has been the analogue of choice for production of nucleotide-bound crystals of ABC proteins, but in some cases the structure resembled the ADP-bound monomeric form (53) and in others the ATP-bound dimer (15). It was previously reported that AMP-PNP inhibited Pgp-mediated ATP hydrolysis at a relatively high concentration, and it was suggested to have a 10-fold lower affinity for binding compared with ATP (30). In contrast, we show that Pgp binds this non-hydrolyzable analogue with affinity comparable with that of other nucleotides and displays competitive inhibition of ATP hydrolysis with a \(K_i\) in the same range. ATP\(\gamma\)S is clearly different from the other non-hydrolyzable analogues in mimicking ATP and promoting formation of the correct dimer interface, likely because it has an unchanged \(\beta\gamma\)-phosphodiester bond. The potential for hydrolysis of nucleotide, even at a very low rate, may be necessary to produce the asymmetric occluded state. The failure of AMP-PNP and AMP-PCP to produce this state may arise because they have no potential for hydrolysis. ATP\(\gamma\)S, but not AMP-PNP, was found to mimic ATP binding to the NBD1 of MRP1 by stimulating ADP trapping at NBD2 (54), and 5 \(\mu M\) ATP\(\gamma\)S competed for azido-ATP photolabeling of the NBDs of MRP1, whereas >20-fold higher concentrations of AMP-PNP were required (55).

The rate of ATP hydrolysis is known to be the rate-limiting step for a bacterial ABC transporter (16), and this is also likely to be true for mammalian ABC exporters such as Pgp. The asymmetric occluded state can likely be detected in the presence of ATP\(\gamma\)S because the rate of hydrolysis is very slow, thus allowing this intermediate to accumulate. In the presence of nucleotides that are hydrolyzed at a high rate (such as ATP, 8-azido-ATP, etc.), the occluded intermediate progresses very rapidly to the transition state, and it is not detectable.

When one molecule of ATP\(\gamma\)S is occluded while the other is bound loosely, Pgp must adopt an asymmetric conformation. We monitored conformational changes in Pgp on ATP\(\gamma\)S binding by collisional quenching of Trp residues. Negatively charged I\(^-\) clearly showed two classes of Trp residues, indicating the induction of heterogeneity, possibly arising from an asymmetric protein conformation. The larger group of Trp residues displayed reduced quenching relative to nucleotide-free or ATP-bound Pgp, indicating that they are less accessible to I\(^-\) after ATP\(\gamma\)S binding. Thus, ATP\(\gamma\)S binding and occlusion in one NBD either sterically blocks the quencher from gaining access to these Trp residues or reduces the positive charge around them. On the other hand, the second smaller group of Trp residues showed increased quenching compared with nucleotide-free or ATP-bound Pgp, implying that conformational rearrangement as a result of ATP\(\gamma\)S occlusion opens up access of quencher to some Trp residues or makes their local environment more positively charged. Of note, 3 of the 11 Trp residues in Pgp are located within or close to the NBDs (42), and these residues may be most affected by nucleotide occlusion.

The unoccupied NBD in vanadate-trapped Pgp was still able to bind nucleotide despite its lack of catalytic activity (35); thus, this state represents an asymmetric (but inactive) post-hydrolysis conformation. Interestingly, the vanadate-trapped state bound ATP\(\gamma\)S at the vacant NBD with high affinity similar to that observed in the occluded conformation. Thus, the low affinity catalytic site that did not carry out ATP hydrolysis appears to switch its conformation to high affinity immediately after catalytic turnover. We speculate that the other NBD containing trapped vanadate may be in the low affinity conforma-
ABC proteins by Higgins and Linton (60) proposed that ATP-induced formation of NBD dimers is the “power-stroke” for substrate transport. However, our results indicate that ATPγS-mediated closure of the dimer interface and nucleotide occlusion do not alter drug binding affinity, implying that drug transport is not driven by ATP binding or occlusion and takes place at a later stage of the catalytic cycle. Senior proposed that drug transport is coupled to relaxation of a high chemical potential conformation of the catalytic site containing bound Mg$^{2+}$-ADP$_2$, which is generated by the process of ATP hydrolysis itself, rather than being coupled to nucleotide binding and occlusion (10, 11). Our data are compatible with this proposal. The processive, clamp model put forward by Tampé and co-workers (61) for the mitochondrial ABC protein Mdl1 proposes that both ATP molecules are hydrolyzed sequentially in the same catalytic cycle, after which the two NBDs dissociate and release ADP. Such a model predicts the existence of several symmetric intermediates during catalysis, in contrast to the alternating sites proposal. In addition, both NBD dimer interfaces are open simultaneously at the end of the dual hydrolysis cycle.

We have shown in this work that progression of the conformation of Pgp containing two bound nucleotides to the asymmetric occluded state does not depend on binding of transport substrate (as proposed by the ATP switch model (60)). Indeed, this is expected, because Pgp has an uncoupled cycle and can hydrolyze ATP efficiently in the absence of drugs. In addition, a thermodynamic study of isolated NBD subunits of a bacterial ABC protein showed that NBD dimer formation is itself a facile and energetically favorable reaction in the absence of the transmembrane domains and transport substrate (62). In Fig. 7, we propose a site-switching model in which the two NBDs alternate in catalysis, and all intermediates are asymmetric to preserve memory between catalytic cycles. The symmetric state (shown outside the catalytic cycle) consists of two ATP molecules bound to Pgp with low affinity, with two open dimer interfaces. This state has been characterized in the case of catalytically arrested MIANS-Pgp or native Pgp bound to poorly hydrolyzed fluorescent and spin-labeled ATP analogues (32, 35, 63). When catalytic cycling starts, one of the two ATP molecules becomes tightly bound, or occluded, thus closing the dimer interface at NBD1. This ATP molecule is then commit-
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ted to enter the transition state and undergo hydrolysis. Drug binding is shown as following ATP binding in Fig. 7, but it is known that these two association steps may occur in a random fashion and are not ordered (32). Hydrolysis of the tightly bound ATP has two consequences; energy is provided for drug transport across the membrane, and the nucleotide binding affinity of the two catalytic sites is simultaneously switched. The dimer interface at NBD1 opens again, allowing the now loosely bound ADP and P, products to dissociate, and the ATP molecule at NBD2 is now tightly bound/occluded at the closed dimer interface. Thus, drug movement across the membrane is driven by ATP hydrolysis, as originally proposed by Senior and co-workers (8, 11), based on the large drop in free energy that accompanies dissociation of P, from the protein after catalytic turnover. ADP dissociates after P, and is replaced by ATP; nucleotide exchange is driven by the high cellular ATP:ADP ratio, as their Pgp binding affinities are similar. At this point Pgp has again achieved the asymmetric conformation with one tightly bound and one loosely bound ATP, and the second catalytic cycle proceeds with hydrolysis at NBD2.

This model proposes ATP site affinity switching coincident with or immediately after ATP hydrolysis, so that one of the two NBD dimer interfaces is always in the tightly bound occluded state at all times. The NBD dimer, thus, never dissociates during catalytic turnover (only one-half of the interface opens after hydrolysis of an ATP molecule), and the asymmetry of the structure is maintained continuously throughout the transport cycle. Because of the high degree of structural similarity between the NBDs of many ABC transporters, the mechanism of energy utilization by these proteins is probably conserved, and this model may be applicable to many members of this family.

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