Site-Directed Fluorescence Labeling of P-Glycoprotein on Cysteine Residues in the Nucleotide Binding Domains†

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ABSTRACT: P-Glycoprotein is a member of the ABC superfamily of membrane transporters, and functions as an ATP-driven active efflux pump for natural products and chemotherapeutic drugs. Overexpression of P-glycoprotein is a major cause of multidrug resistance in human cancers. Sulfhydryl modification agents are known to inactivate both P-glycoprotein ATPase activity and transport function. In the present study, P-glycoprotein purified from CH^RB30 cells was covalently labeled at two conserved Cys residues, one within each of the nucleotide binding domains, using 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS). MIANS modification inactivated P-glycoprotein ATPase function, in a concentration-dependent fashion. Increasing concentrations of ATP blocked MIANS labeling with an IC_{50} of 0.37 mM (similar to the K_M for ATP hydrolysis), which suggests that the label is located close to the site of ATP binding within the nucleotide binding domain. A blue shift in the fluorescence spectrum of MIANS bound to P-glycoprotein indicated that the labeled Cys residues are situated in a nonpolar environment. MIANS-labeled P-glycoprotein was still able to bind ATP, as demonstrated by quenching of the fluorescence, with a K_d of 0.46 mM. Addition of a variety of drugs and chemosensitizers to MIANS-labeled P-glycoprotein led to substantial quenching of the probe fluorescence within the nucleotide binding domains. Dissociation constants for drug binding measured by fluorescence quenching were in the range of 0.77 μM for vinblastine to 158 μM for colchicine. Quenching by ATP and drugs was independent and additive, suggesting that each produces a defined change in the protein. The rate of MIANS labeling of Pgp was reduced in the presence of drugs and chemosensitizers, implying that a long-range conformational change arises from drug binding which alters the accessibility of the nucleotide binding domains to MIANS. These results suggest that there is conformational communication between the drug binding site(s) of P-glycoprotein and the ATPase catalytic sites within the nucleotide binding domains.

P-Glycoprotein (Pgp)[1] is a member of the ABC (ATP binding cassette) superfamily of membrane transporters. Proteins within this family are characterized by an internal tandem duplication, with six membrane-spanning segments and one nucleotide binding domain (NBD) within each half. Overexpression of Pgp is associated with resistance to a broad spectrum of hydrophobic, natural product drugs, so-called multidrug resistance (MDR) [for reviews, see Gottesman and Pastan (1993) and Georges et al. (1990)]. Pgp is believed to be a major cause of drug resistance in human cancers. Reconstitution studies have shown that Pgp transports a variety of hydrophobic drugs up a concentration gradient, powered by ATP hydrolysis at the nucleotide binding domains (Sharom et al., 1993; Sharom, 1995; Shapiro & Ling, 1995).

Purified Pgp exhibits high levels of constitutive ATPase activity (Urbatsch et al., 1994; Shapiro & Ling, 1994; Sharom et al., 1995a; Senior et al., 1995). The ATPase activity of membrane-bound Pgp is stimulated severalfold by various MDR spectrum drugs, chemosensitizers, and hydrophobic peptides (Al-Shawi & Senior, 1993; Sharom et al., 1995b; Scarborough, 1995). The activity of purified Pgp in detergent solution is also increased by drugs, although often to a lesser extent (Urbatsch et al., 1994; Sharom et al., 1995a). These observations indicate that some sort of communication or coupling must exist between the drug binding site(s) and the catalytic sites for ATP hydrolysis within the NBDs. The drug binding sites of Pgp are widely believed to reside within the membrane-spanning helices (Raviv et al., 1990; Dhir et al., 1993; Loo & Clarke, 1993a,b), whereas the NBDs appear to be separately folded, soluble protein domains. As yet, little is known concerning the nature or extent of the coupling between these two regions of the transporter.

Both the transport function (Doige & Sharom, 1992) and ATPase activity (Doige et al., 1992; Al-Shawi & Senior, 1993; Urbatsch et al., 1994) of Pgp are abolished by sulfhydryl reagents, including *N*-ethylmaleimide (NEM), *p*-(chloromercuri)benzoate, and $HgCl_2$. Senior and co-workers showed that two reactive Cys residues are modified by NEM, one within each half of the Pgp molecule (Al-Shawi et al., 1994). NEM modification can be blocked by the presence of ATP (Al-Shawi et al., 1994; Urbatsch et al.,

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTE, dithioerythritol; MDR, multidrug resistance; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid; NBD, nucleotide binding domain; NEM, *N*-ethylmaleimide; Pgp, P-glycoprotein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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1994), which suggests that the two modified positions are close to the catalytic sites within the NBDs, and may play an important role in ATP binding or hydrolysis.

Human Pgp contains a total of seven Cys residues. Loo and Clarke (1995a) used site-directed mutagenesis to replace all the Cys residues in Pgp with Ala, and showed that the Cys-less protein was still able to confer a high level of drug resistance (63−81% of wild-type, depending on the drug tested). Therefore, Cys residues are not necessary for either ATP hydrolysis or drug transport. However, Cys-less Pgp was no longer subject to inhibition by NEM. Loo and Clarke (1995b) further demonstrated that reinsertion of only two Cys residues into the Cys-free Pgp restored sensitivity to NEM inactivation. The two Cys residues responsible for conferring NEM sensitivity, residues 431 and 1074 in the human sequence, are located within the Walker A consensus sequences of each NBD, and are highly conserved in all mammalian Pgps. Although these two Cys residues are not necessary for Pgp function, they are likely close to the catalytic sites. Modification of these residues with a bulky maleimide group may lead to inhibition of ATPase activity via a steric effect. This situation would be similar to that observed for lactose permease; Cys 148 of the permease displays similar properties, and has been shown to be part of the substrate binding site (Jung et al., 1994).

We recently reported the purification of Pgp with very high constitutive ATPase activity from MDR CH^RC5 Chinese hamster ovary cells (Sharom et al., 1995a), using a two-step detergent extraction, followed by lectin affinity chromatography. Further improvements have been made to this procedure, which permit purification of Pgp on a scale large enough for physical methods to be used to probe the protein. In this study, we have covalently labeled purified Pgp at the two reactive Cys residues, one within each NBD, using the sulfhydryl-reactive fluorescence probe 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS). Results of fluorescence quenching experiments indicate that MIANS-modified Pgp retains ATP binding activity, and provide evidence for conformational coupling between the NBDs and the drug binding site(s) on Pgp.

MATERIALS AND METHODS

Materials. Asolectin was obtained from Fluka (Ronkonkoma, NY). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), NEM, dithioerythritol (DTE), Na_2-ATP, drugs, and chemosensitizers were purchased from Sigma Chemical Co. (St. Louis. MO). MIANS was supplied by Molecular Probes (Eugene, OR).

MDR Cell Lines and Plasma Membrane Preparation. The MDR Chinese hamster ovary cell line CH^RB30 (Ling & Thompson, 1974) was grown as described previously (Loe & Sharom, 1993, 1994). Cells were cultured in the presence of 30 μg/mL colchicine at 37 °C in a humidified atmosphere of 5% CO_2 in α-minimal essential medium supplemented with 10% heat-inactivated iron-supplemented/defined bovine calf serum (Hyclone Laboratories, Logan, UT), penicillin (1000 units/mL), streptomycin (1 mg/mL), and 2 mM L-glutamine. Cell culture supplies were purchased from Gibco Canada (Burlington, ON). Plasma membrane vesicles were isolated from CH^RB30 cells as described previously (Doige & Sharom, 1991). Protein was quantitated by the method of Bradford (1976) for plasma membrane, and by the method of Peterson (1983) for purified Pgp, using bovine serum albumin (crystallized and lyophilized, Sigma) as a standard. Membrane vesicles were stored at −70 °C for no more than 3 months before use.

Pgp Purification. Pgp was purified from CH^RB30 plasma membrane by a modification of the method previously described for CH^RC5 (Sharom et al., 1995a). Following the initial extraction of plasma membrane with CHAPS, the S_1 pellet was solubilized at a final protein concentration of 1 mg/mL in 2 mM CHAPS buffer. Incubation and centrifugation resulted in a soluble CH^RB30 S_2 supernatant which was highly enriched in Pgp. Contaminating glycoproteins were removed from the S_2 fraction by a single chromatographic run through a column of either lentil lectin−Sepharose or concanavalin A−Sepharose (Pharmacia). The final product consisted of a solution of 90−95% pure Pgp, at a concentration of 0.1−0.2 mg/mL, in 2 mM CHAPS/50 mM Tris-HCl/0.15 M NH_4Cl/5 mM $MgCl_2$/0.02% NaN_3, pH 7.5. SDS−PAGE was carried out as described previously (Sharom et al., 1995a). The Pgp preparation was kept on ice and used within 24 h.

Measurement of Pgp ATPase Activity. Mg^{2+}-ATPase activity of Pgp was determined by measuring the release of inorganic phosphate from ATP, as described previously (Doige et al., 1992; Sharom et al., 1995a), in the presence of 1 mM ATP and 5 mM Mg^{2+}. To quantitate inhibition of Pgp ATPase, various concentrations of NEM or MIANS were preincubated for 10 min at 22 °C with Pgp. Unreacted NEM/MIANS was quenched with 1 mM DTE before initiation of the assay by addition of ATP.

MIANS Labeling of Pgp and Fluorescence Measurements. MIANS was dissolved at 2.5 mM in methanol, and the actual concentration was determined using absorbance measurements (λ_{max} = 322 nm, ϵ = 17 000 M^{-1} cm^{-1}; Haugland, 1989). Fluorescence was measured at 22 °C using a PTI Alphascan-2 spectrofluorimeter (Photon Technology Inc., South Brunswick, NJ). Emission spectra were recorded using an excitation wavelength of 322 nm and 4 nm slits for excitation and emission.

To determine the rate of MIANS reaction with Cys residues in Pgp, the protein (50 μg/mL in 2 mM CHAPS) was preincubated with either buffer or increasing concentrations of ATP, drug, or chemosensitizer, at 22 °C for 5 min. Labeling was initiated by addition of 4 μL of 0.5 mM MIANS to 500 μL of Pgp solution, to give a final concentration of 4 μM, and fluorescence was monitored continuously at an emission wavelength of 420 nm.

To prepare fully modified Pgp, purified protein (100 μg/mL in 2 mM CHAPS) was incubated with 20 μM MIANS at 22 °C for 1 h in the dark. Unreacted MIANS was quenched with 1 mM DTE, and the modified Pgp was separated by gel filtration on a pre-filled 10 mL column of Bio-Gel P-6 (Bio-Rad Laboratories, Mississauga, ON) equilibrated with 2 mM CHAPS buffer. The final concentration of MIANS-labeled Pgp was 50 μg/mL. As a control, MIANS was reacted with the soluble sulfhydryl-containing molecule, DTE.

Stoichiometry of MIANS Labeling. To determine the stoichiometry of labeling, Pgp in 2 mM CHAPS (100 μg/mL) was treated with increasing concentrations of MIANS for 20 min at 22 °C to obtain different levels of ATPase inhibition. Unreacted MIANS was quenched with DTE, and samples were dialyzed against 3 × 1 L of 50 mM ammonium

bicarbonate at 4 °C in the dark. The Pgp samples were then lyophilized and redissolved in 6 M guanidine hydrochloride at a concentration of 200−300 μg/mL. Covalently bound MIANS was quantitated by the absorbance measurement at 322 nm, using free MIANS to generate a standard curve, and unlabeled Pgp as a blank. Protein concentration was determined according to Peterson (1983). The molar mass of Pgp polypeptide used for stoichiometry calculations was 140 kDa.

Fluorescence Quenching Studies. Fluorescence quenching studies with ATP and various drugs and chemosensitizers were carried out on solutions of 50 μg/mL Pgp in 2 mM CHAPS buffer at 22 °C, in the presence of 0.5 mg/mL asolectin (soybean phospholipids). Phospholipids were added as large unilamellar vesicles, which were prepared by extrusion through 100 nm polycarbonate filters (Hope et al., 1985; Mayer et al., 1986). The working solutions of ATP, and various drugs and chemosensitizers, were also prepared in 2 mM CHAPS/0.5 mg/mL asolectin. Quenching experiments were performed by successively adding 5 μL aliquots of ATP or drug solution to 500 μL of MIANS-labeled Pgp in a 0.5 cm quartz cuvette. After each addition, the steady-state fluorescence was measured at 420 nm. Fluorescence intensities were corrected for dilution, scattering, and the inner filter effect, using the equation (Parker, 1968; Lakowicz, 1983):

$$F_{\mathrm{icor}} = (F_{\mathrm{i}} - B)(V_{\mathrm{i}}/V_{\mathrm{o}})10^{0.5b(A_{\lambda\mathrm{ex}}+A_{\lambda\mathrm{em}})}$$

where F_{icor} is the corrected value of the fluorescence intensity at a given point in the titration, F_{i} is the experimentally measured fluorescence intensity, B is the background fluorescence intensity (caused mainly by lipid vesicle scattering), V_{o} is the initial volume of the sample, V_{i} is the volume of the sample at a given point in the titration ($V_{\mathrm{i}}/V_{\mathrm{o}}$ is the dilution factor), b is the path length of the optical cell in centimeters, and $A_{\lambda\mathrm{ex}}$ and $A_{\lambda\mathrm{em}}$ are the absorbances of the sample at the excitation and emission wavelengths, respectively.

The experimental data were computer-fitted to the equation:

$$(\Delta F/F_0 \times 100) = \frac{(\Delta F_{\mathrm{max}}/F_0 \times 100)[\mathrm{S}]}{K_{\mathrm{d}} + [\mathrm{S}]}$$

where ($\Delta F/F_0 \times 100$) is the percent quenching (percent change in fluorescence relative to the initial value) following addition of drug or ATP at a concentration [S], and K_{d} is the dissociation constant. Fitting was carried out using nonlinear regression with the Marquardt−Levenberg algorithm (SigmaPlot for Windows, Jandel Scientific), and values of K_{d} and ($\Delta F_{\mathrm{max}}/F_0 \times 100$) were extracted. The data were also displayed as a double-reciprocal plot of $1/(\Delta F/F_0)$ *vs* 1/[ATP] or 1/[drug].

RESULTS

Purification of ATPase-Active Pgp from CH^{R}B30 Cells. We previously reported the purification of Pgp to >90% purity using a two-step selective extraction of plasma membrane from CH^{R}C5 cells with CHAPS, followed by two sequential chromatographic runs through lentil lectin−Sepharose to remove contaminating glycoproteins (Sharom et al., 1995a). Further improvements have been made to this procedure, primarily by the use of the very highly drug-resistant cell line CH^{R}B30 [680-fold resistant to colchicine; see Loe and Sharom (1993)], which was originally derived from CH^{R}C5 (96-fold resistant). Plasma membrane from CH^{R}B30 contains substantially higher levels of Pgp when compared to CH^{R}C5. When the two-step CHAPS extraction was carried out with CH^{R}B30 plasma membrane, >70% of the total ATPase activity was recovered in the S_2 fraction (Table 1), whose specific activity was almost double that of S_2 from CH^{R}C5 membrane (Sharom et al., 1995a). We previously showed that Pgp was the only ATPase present in the S_2 fraction from MDR Chinese hamster ovary cells (Doige et al., 1992). SDS−PAGE showed that Pgp was the major protein band in the S_2 fraction from CH^{R}B30 cells (Figure 1, lane 2). A single chromatography step on lentil lectin−Sepharose, rather than the two steps used previously (Sharom et al., 1995a), was sufficient to remove contaminants from S_2, and the Pgp obtained was >90−95% pure (Figure 1, lane 3). We also determined that a single run through concanavalin A−Sepharose gave very similar results (Table 1), and has the advantage of being considerably cheaper. The overall enrichment in ATPase specific activity was approximately 6.7-fold, since we estimate that ∼80% of the total ATPase activity of the plasma membrane starting material is attributable to Pgp. This value is in agreement with the enrichment of Pgp protein (as estimated by

Table 1: Purification of Pgp from CH^{R}B30 Cells[a]

stage of purification	Mg^{2+}-ATPase sp act. [μmol min^{-1} (mg of protein)$^{-1}$]	total ATPase act. (μmol/min)	protein (mg)
CH^{R}B30 plasma membrane	0.421 ± 0.007	0.842 (100)	2.00 (100)
CHAPS extract (S_2 fraction)	0.926 ± 0.027	0.597 (71)	0.645 (32)
lentil lectin run-through	2.248 ± 0.022	0.506 (60)	0.225 (11)
concanavalin A run-through	2.116 ± 0.030	0.504 (60)	0.238 (12)

[a] Samples from various stages of the purification procedure were assayed for Mg^{2+}-dependent ATPase activity and protein content as described under Materials and Methods. The CHAPS extract was further purified by passage through a column of either lentil lectin−Sepharose or concanavalin A−Sepharose. The values in parentheses represent the percent recovery of the ATPase activity and total protein, starting from CH^{R}B30 plasma membrane.

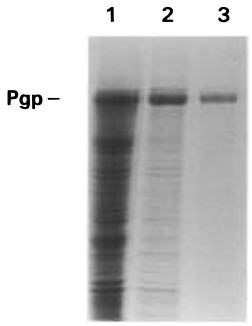

FIGURE 1: Purification of Pgp from MDR CH^{R}B30 cells. Samples were dialyzed against 50 mM ammonium bicarbonate, lyophilized, and then analyzed by SDS−PAGE followed by staining with Coomassie Blue. Lane 1, CH^{R}B30 plasma membrane, 24 μg of protein; lane 2, CHAPS extract (S_2 fraction), 6 μg of protein; lane 3, lentil lectin−Sepharose run-through, 2.4 μg of protein. The Pgp band at 170−180 kDa is indicated by a dash.

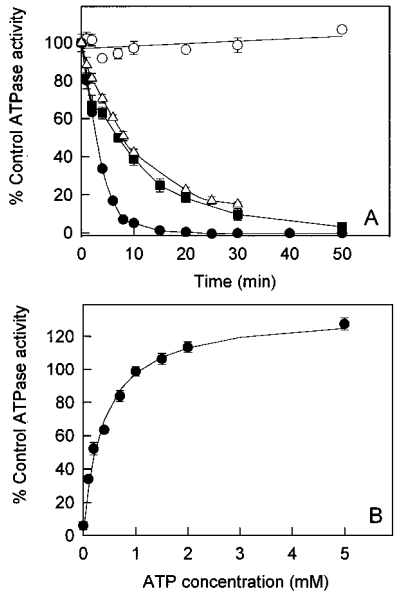

FIGURE 2: Time course of inactivation of Pgp ATPase activity by NEM and MIANS, and protection by ATP. (A) Purified Pgp (in 2 mM CHAPS buffer) was treated with 4 μM MIANS or 10 μM NEM, and the ATPase activity was determined at various times, after quenching of unreacted MIANS/NEM with DTE. Percent control ATPase values were calculated relative to untreated Pgp, and represent means for duplicate determinations. Conditions were as follows: NEM at 22 °C (■); MIANS at 22 °C (●); MIANS at 0 °C (△); MIANS at 22 °C in the presence of 2 mM ATP (○). (B) Protection of Pgp from MIANS inactivation by ATP. Purified Pgp (in 2 mM CHAPS buffer) was treated with 4 μM MIANS at 22 °C in the presence of increasing concentrations of ATP. The ATPase activity was determined after quenching of unreacted MIANS/NEM with DTE.

Coomassie Blue staining) from ~15% in CH^{R}B30 plasma membrane to purified Pgp. The specific activity of the final product was 2.1−2.2 μmol min^{-1} (mg of protein)$^{-1}$, which is the highest basal ATPase activity reported to date for purified Pgp. The overall yield of Pgp from CH^{R}B30 plasma membrane was ~120 μg/mg of membrane protein, over twice that obtained from CH^{R}C5 cells. The entire purification procedure, starting from plasma membrane, can be carried out in less than 4 h. The Pgp preparation is relatively concentrated (0.1−0.2 mg/mL in 2 mM CHAPS buffer), and is free of added lipids, ATP, and salts, making it well-suited for direct use in physical studies.

Inactivation of Pgp ATPase Activity by NEM and MIANS. The ATPase activity of purified Pgp was reduced in a time-dependent fashion by treatment with NEM (Figure 2A). At 22 °C, the sulfhydryl-reactive fluorescent probe MIANS also inhibited the Pgp ATPase. The rate of inactivation by 4 μM MIANS was substantially higher than that observed for 10 μM NEM (Figure 2A), and modification of Pgp was essentially complete after 20 min under these conditions. Even at 0 °C, MIANS reacted with Pgp quite rapidly. Pgp ATPase activity could be almost completely protected from MIANS inhibition by the presence of 2 mM ATP (Figure 2A), but not by AMP (data not shown). Additional MIANS labeling experiments were carried out in the presence of increasing concentrations of ATP. As shown in Figure 2B, ATP blocked MIANS inactivation of Pgp in a concentration-dependent fashion. The ATP concentration which resulted in half-maximal protection (IC_{50}) was 0.37 mM, which is very close to the K_M for ATP hydrolysis previously reported for purified Pgp in CHAPS (0.4 mM; Sharom et al., 1995a).

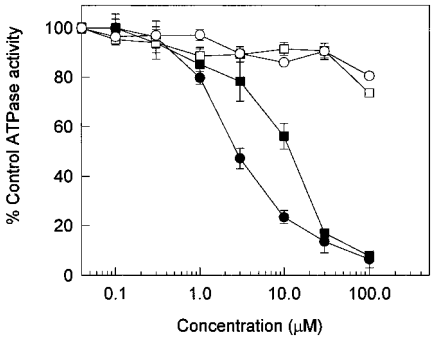

FIGURE 3: Concentration-dependence of inactivation of Pgp ATPase activity by NEM and MIANS at 22 °C, and protection by ATP. Purified Pgp (in 2 mM CHAPS buffer) was treated with increasing concentrations of NEM (■) or MIANS (●) for 10 min at 22 °C, and the ATPase activity was determined after quenching of unreacted MIANS with DTE. Percent control ATPase values were calculated relative to untreated Pgp, and represent means for duplicate determinations. Open symbols indicate the results of the same experiment, carried out in the presence of 2 mM ATP.

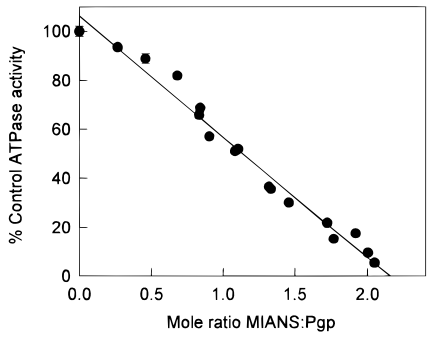

FIGURE 4: Stoichiometry of MIANS labeling of Pgp. Purified Pgp (100 μg/mL in 2 mM CHAPS buffer) was treated with increasing concentrations of MIANS to obtain different levels of ATPase inhibition. Following quenching of unreacted MIANS with DTE, dialysis, and lyophilization, the labeled Pgp samples were redissolved in 6 M guanidine hydrochloride. The amount of bound MIANS in each sample was quantitated by absorbance measurements at 322 nm. Data points are the means of duplicate determinations.

These results suggest that the Cys residues modified by MIANS lie close to the sites for ATP binding and hydrolysis within the NBDs of Pgp.

Inactivation of Pgp ATPase by both MIANS and NEM was concentration-dependent (Figure 3). MIANS treatment generally resulted in a higher level of ATPase inactivation compared to NEM at the same concentration, except at the high end of the range tested, where inhibition was essentially complete for both compounds. The presence of ATP at a concentration of 2 mM again blocked inactivation over the entire concentration range (Figure 3). The stoichiometry of labeling was determined by inactivating Pgp ATPase to varying degrees with different MIANS concentrations, followed by quantitation of the bound chromophore using absorbance spectroscopy. As shown in Figure 4, complete inhibition of the ATPase activity corresponded to the covalent incorporation of 2 mol of MIANS per mole of Pgp.

Fluorescent Labeling of Pgp with MIANS. MIANS only becomes fluorescent after the maleimide moiety undergoes covalent reaction with sulfhydryl groups (Haugland, 1989). This feature allows the time-course of labeling to be conveniently followed by continuous monitoring of the fluorescence. Addition of MIANS to purified Pgp led to a rapid increase in fluorescence emission intensity (Figure 5A);

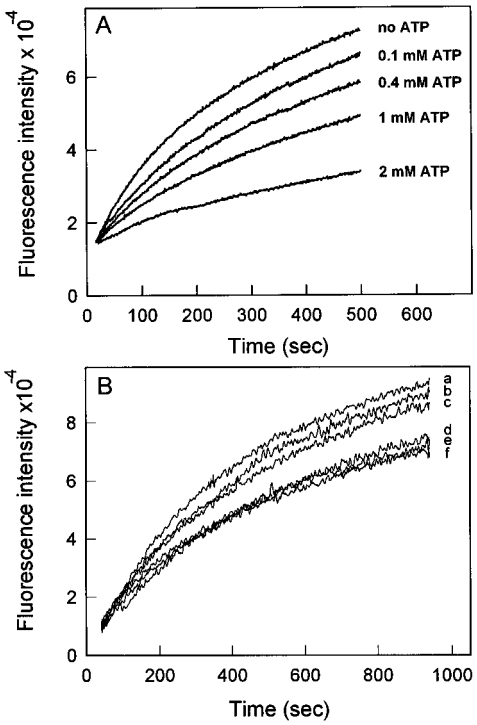

FIGURE 5: Reaction of Pgp with the fluorescence probe MIANS. MIANS labeling was carried out using 50 μg/mL purified Pgp (500 μL, in 2 mM CHAPS buffer). Pgp was preincubated for 5 min at 22 °C with either buffer, or various concentrations of ATP (A), or drugs and chemosensitizers (B). Labeling was initiated by addition of MIANS to a final concentration of 4 μM. Fluorescence emission was recorded continuously at 420 nm. Drugs and chemosensitizers used in (B) were (a) none, (b) 10 μM trifluoperazine, (c) 2 μM vinblastine, (d) 10 μM verapamil, (e) 10 μM doxorubicin, and (f) 50 μM colchicine.

the rate of increase in fluorescence declined smoothly over a period of about 8 min. Preincubation with ATP resulted in a reduction in the rate of reaction of Pgp with MIANS, which was dependent on the added ATP concentration (Figure 5A). If Pgp was first reacted with NEM, no increase in fluorescence was seen on addition of MIANS, indicating that the two agents compete for reaction with the same sites on the transporter. Complete labeling of Pgp was achieved by incubation of the protein with 20 μM MIANS for 1 h at 22 °C, followed by quenching of unreacted MIANS with DTE, and separation of the labeled Pgp by gel filtration chromatography.

When Pgp was incubated with MIANS in the presence of various drugs and chemosensitizers, a substantial reduction in the rate of labeling was observed (Figure 5B). The magnitude of the decrease varied with the compound under study, being largest for colchicine and smallest for trifluoperazine. These results suggest that binding of drugs and chemosensitizers to Pgp creates a change in the conformation of the protein, which in turn alters the accessibility of the two Cys residues within the NBDs to MIANS.

The MIANS probe is known to be highly sensitive to the polarity of its immediate surroundings (Haugland, 1989; Ksenzenko et al., 1993), with the emission maximum exhibiting a blue shift in a nonpolar environment. The fluorescence emission spectrum of MIANS-labeled Pgp is shown in Figure 6A, compared to the spectrum for MIANS following reaction with the soluble sulfhydryl compound, DTE. The emission maximum for MIANS−DTE is at about 450 nm, whereas that for MIANS−Pgp is at about 420 nm.

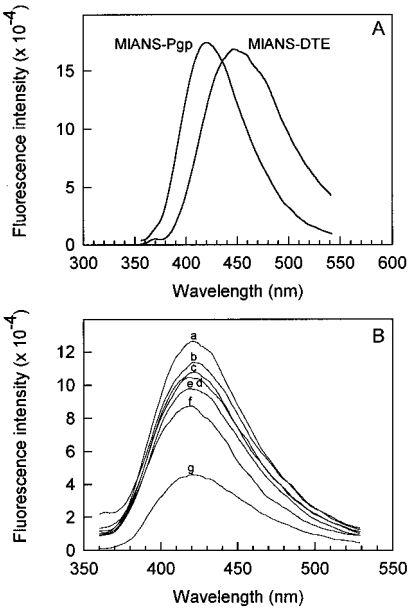

FIGURE 6: Fluorescence emission spectra of MIANS-labeled Pgp, and the effect of drugs and chemosensitizers. (A) Fluorescence spectrum of MIANS (λ_{ex} = 322 nm, λ_{em} = 420 nm) following reaction with Pgp, and the soluble compound DTE. (B) Fluorescence spectrum of MIANS-labeled Pgp in the absence of drugs (a), and following addition of 10 μM reserpine (b), 10 μM verapamil (c), 5 μM vinblastine (d), 2 mM ATP (e), 25 μM doxorubicin (f), and 50 μM colchicine (g). For all of the drugs listed above, the concentrations tested were close to saturating, based on the K_d values measured by fluorescence quenching titration, with the exception of colchicine, which has a high K_d. In this case, quenching could not be readily saturated in the experimentally accessible concentration range.

Such a large blue shift indicates that the labeled Cys residues in Pgp reside in a relatively hydrophobic environment.

Quenching of MIANS-Labeled Pgp by ATP. We have shown that purified Pgp is highly dependent on phospholipids for function (Doige et al., 1993; Sharom et al., 1995a). Further experiments on MIANS−Pgp were, therefore, carried out in the presence of soybean phospholipids (asolectin), to assist the protein in adopting a close-to-native conformation. The presence of 0.5 mg/mL phospholipids did not lead to any change in the λ_{max} of the bound MIANS probe. Addition of ATP to MIANS-labeled Pgp resulted in a large quenching of the fluorescence (Figure 6B, trace e), indicating that Pgp is still able to bind ATP after modification of the two Cys residues within the NBDs. Nucleotide binding alters the local environment of the fluorescence probe, changing its quantum yield. There was no significant shift in the MIANS emission maximum on ATP addition. A fluorescence quenching titration was then carried out, measuring the degree of quenching of the MIANS fluorescence following addition of increasing concentrations of ATP. ATP-induced quenching of MIANS−Pgp was concentration-dependent and saturable, following a typical hyperbolic binding curve (Figure 7A). The ATP quenching data are displayed as a double-reciprocal plot in Figure 7B. The data were fitted to an equation for interaction with a single type of binding site (see Materials and Methods). The K_d value for ATP binding was estimated to be 0.46 mM (Table 2), which is very similar to the K_M for ATP hydrolysis by purified Pgp (0.4 mM; Sharom et al., 1995a).

Quenching of MIANS-Labeled Pgp by Drugs and Chemosensitizers. Addition of various MDR spectrum drugs and

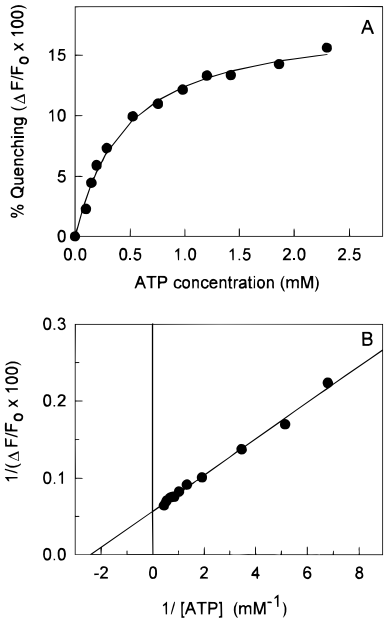

FIGURE 7: Quenching of fluorescence of MIANS-labeled Pgp by ATP. Increasing concentrations of ATP were added to 50 μg/mL MIANS-labeled Pgp (in 2 mM CHAPS buffer) at 22 °C in the presence of 0.5 mg/mL asolectin, and the fluorescence emission at 420 nm was recorded. (A) The percent quenching of fluorescence ($\Delta F/F_0 \times 100$) was calculated relative to the fluorescence of MIANS-labeled Pgp in the absence of ATP, at increasing ATP concentrations. The continuous line represents the best computer-generated fit of the data points (shown by the symbols) to an equation describing interaction of ATP with a single type of binding site (see Materials and Methods). (B) Data are displayed as a double-reciprocal plot of $1/(\Delta F/F_0 \times 100)$ vs 1/[ATP].

Table 2: Parameters of Fluorescence Quenching of MIANS-Labeled Pgp[a]

ligand	K_d (μM)	$(\Delta F_{max}/F_0 \times 100)$ (%)
ATP	460 ± 0.04	18.1 ± 0.5
reserpine	0.73 ± 0.08	11.0 ± 0.32
vinblastine	0.77 ± 0.03	17.3 ± 0.03
verapamil	2.4 ± 0.07	14.7 ± 0.11
doxorubicin	4.4 ± 0.6	28.6 ± 1.5
trifluoperazine	7.6 ± 0.9	24.9 ± 1.06
daunorubicin	10.5 ± 1.4	52.8 ± 3.2
colchicine	158 ± 9	181 ± 6

[a] Experimental quenching data, of the type shown in Figure 8, were computer-fitted to an equation describing interaction with a single type of binding site using nonlinear regression (see Materials and Methods). Values of K_d and $\%(\Delta F_{max}/F_0)$ shown are the means extracted by computer-fitting from at least two separate independent experiments using different batches of MIANS-labeled Pgp.

chemosensitizers also led to substantial quenching of the bound MIANS probe. The degree of quenching varied with the particular drug added (Figure 6B); for example, colchicine caused a high level of quenching, whereas quenching was at a relatively low level in the presence of saturating concentrations of verapamil. In order to examine drug interactions with Pgp in more detail, fluorescence quenching titrations were carried out, measuring the degree of quenching of MIANS−Pgp fluorescence following addition of increasing concentrations of each drug. As shown in Figure 8A,C for four representative Pgp substrates, two MDR drugs (vinblastine, doxorubicin), and two chemosensitizers (verapamil, reserpine), the changes in fluorescence are concentration-dependent, saturable, and follow a hyperbolic curve.

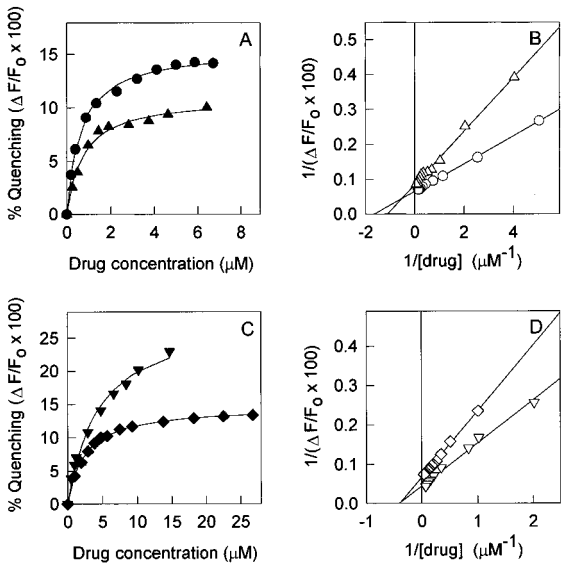

FIGURE 8: Quenching of fluorescence of MIANS-labeled Pgp by drugs and chemosensitizers. Increasing concentrations of various drugs and chemosensitizers were added to 50 μg/mL MIANS-labeled Pgp (in 2 mM CHAPS buffer) at 22 °C in the presence of 0.5 mg/mL asolectin, and the fluorescence emission at 420 nm was recorded. The percent quenching of fluorescence ($\Delta F/F_0 \times 100$) was calculated relative to the fluorescence of MIANS-labeled Pgp in the absence of drugs, at increasing drug concentrations. 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 Materials and Methods); (A) vinblastine (●) and reserpine (▲); (C) verapamil (♦) and doxorubicin (▼). Data are displayed as double-reciprocal plots of $1/(\Delta F/F_0 \times 100)$ vs 1/[drug]; (B) vinblastine (○) and reserpine (△); (D) verapamil (◇) and doxorubicin (▽).

These results suggest that drugs bind directly to Pgp, and that such binding causes an alteration in the quantum yield of the MIANS probe in the NBDs. Since these changes are observed in the absence of ATP, nucleotide binding is clearly not a prerequisite for interaction of drugs with Pgp, or the generation of the drug-induced alteration in the environment of the fluorescence probe.

Curve-fitting was again used to estimate the parameters of drug binding; values of K_d and $\%(\Delta F_{max}/F_0)$, which represents the maximum percent quenching of MIANS−Pgp fluorescence induced by each drug, are shown in Table 2. The fluorescence quenching data for the four drugs are displayed as double-reciprocal plots in Figure 8B,D. The K_d values obtained from fitting of the fluorescence quenching data cover a range of affinities, from very high (0.77 μM for vinblastine) to much lower (158 μM for colchicine). In general, these K_d values are in agreement with the relative "affinities" of these compounds as assessed by other methods, such as their ability to inhibit photoaffinity and chemical affinity labeling of Pgp, and their ability to compete for drug transport (Doige & Sharom, 1992; Sharom et al., 1993). For all of the fluorescence spectra shown in Figure 6B, the drug concentrations were close to saturating (based on the K_d values measured by fluorescence quenching titration, see Table 2), with the exception of colchicine. This compound has a high K_d, and quenching could not be readily saturated in the experimentally accessible concentration range. Most of the drugs listed in Table 2 did not lead to a change in the λ_{max} of fluorescence emission of the bound MIANS (Figure 6B). The two exceptions were daunorubicin, which caused

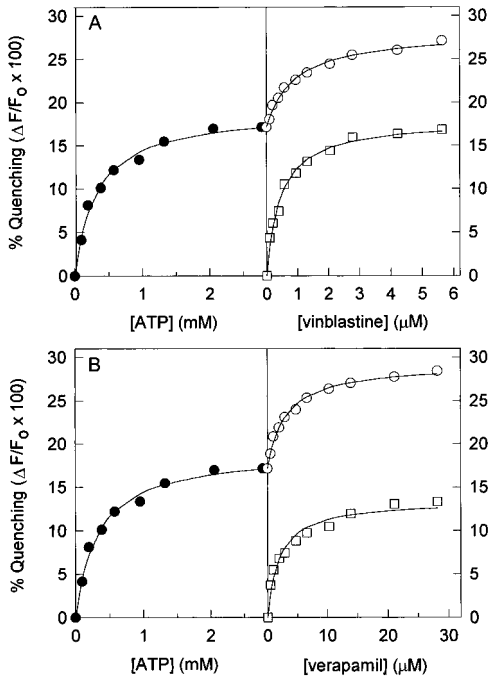

FIGURE 9: Quenching of the fluorescence of MIANS-labeled Pgp by sequential addition of ATP and drugs. Increasing concentrations of ATP were added to 50 μg/mL MIANS-labeled Pgp (in 2 mM CHAPS buffer) at 22 °C in the presence of 0.5 mg/mL asolectin, and the fluorescence emission at 420 nm was recorded (●; left-hand panels of A and B). The same sample was then titrated with increasing concentrations of either vinblastine (A) or verapamil (B), and further changes in fluorescence were recorded (○; right-hand panels of A and B, top sections). The same MIANS-labeled Pgp sample was also titrated with vinblastine or verapamil without prior titration with ATP (□; right-hand panels of A and B, bottom sections). The percent quenching of fluorescence ($\Delta F/F_0 \times 100$) was calculated relative to the fluorescence of MIANS-labeled Pgp in the absence of ATP or drugs. The continuous lines represents the best computer-generated fit of the data points (shown by the symbols) to an equation describing interaction of ATP/drug with a single type of binding site.

a small blue shift of about 5 nm, and trifluoperazine, which produced a red shift of a similar magnitude. The observed wavelength shifts suggest that binding of these two drugs leads to slight changes in the polarity of the environment around the bound MIANS probe.

Further experiments were carried out to determine whether the quenching effects of ATP and drugs were additive, and independent of each other. MIANS-labeled Pgp was first titrated with ATP up to 2.7 mM (Figure 9, left-hand panels), at which point maximal ATP-induced quenching was achieved. The Pgp containing bound ATP was then titrated with increasing concentrations of vinblastine (Figure 9A) or verapamil (Figure 9B); an additional concentration-dependent, saturable quenching component was observed in each case. These results indicate that ATP and drugs/chemosensitizers each produce a defined change in Pgp, and that these changes are independent and additive. In the case of verapamil, the quenching curve seen following ATP titration was virtually indistinguishable from that observed with the same labeled Pgp preparation with no prior ATP titration (Figure 9B). However, the extent of vinblastine quenching was reduced by ~40% when measured after ATP binding (Figure 9A). The K_d values for binding of the two drugs were not significantly different when determined before or after addition of ATP.

DISCUSSION

Previous work in our laboratory initially demonstrated that both the transport function and ATPase activity of Pgp were abolished by treatment with the sulfhydryl-reactive agents NEM, *p*-(chloromercuri)benzoate, and $HgCl_2$ (Doige & Sharom, 1992; Doige et al., 1992). Studies by Senior and co-workers on plasma membranes containing high levels of Pgp, and on purified Pgp, confirmed these observations; they established that two sites on Pgp reacted with NEM, and that ATP protected against modification (Al-Shawi et al., 1994). These results clearly implicated two Cys residues as being located close to the sites of ATP binding and hydrolysis. Loo and Clarke (1995b) further determined that the two NEM-reactive Cys residues are situated within the consensus regions of the Walker A motifs of each NBD, GNSGCGKS and GSSGCGKS, respectively. We have exploited these two residues as a unique target for site-directed labeling of Pgp using the Cys-reactive fluorescence probe MIANS. MIANS has been used to probe conformational changes in many different proteins; the one of most relevance to the present study is the membrane transporter lactose permease. Kaback and co-workers constructed various Cys substitutions in helix V, close to the substrate binding site, labeled each with MIANS, and subsequently explored substrate effects on fluorescence emission spectra and quenching (Wu & Kaback, 1994).

In the present study, purified Pgp with a very high level of ATPase activity has been obtained by further improvements to the purification scheme we reported previously (Sharom et al., 1995a). The Chinese hamster ovary cells used as the Pgp source express primarily (>90%; Al-Shawi et al., 1994) the MDR1 isoform of the multidrug transporter. Because this Pgp preparation is relatively concentrated, and is free of added lipids, ATP, and salts, it has been employed directly in a site-directed fluorescence labeling study using MIANS. MIANS reacted rapidly with Pgp in a very similar fashion to NEM, resulting in the modification of two Cys residues. The two agents compete for the same sites within the protein. The NEM-sensitive Cys in the NBDs of human Pgp are 431 and 1074 (Loo & Clarke, 1995b); the equivalent Cys residues in hamster Pgp are 428 and 1071. Reaction with these two sites within Pgp resulted in progressive inactivation of Pgp ATPase function. ATP provided protection against MIANS inactivation, while AMP did not. The observed concentration dependence of blocking by ATP (IC_{50} = 0.37 mM) is similar to the high K_M for ATP (0.4 mM) reported previously for purified Pgp in CHAPS. Labeling of Pgp with MIANS was followed by monitoring the increase in probe fluorescence with time. Increasing concentrations of ATP again inhibited this fluorescence increase. Taken together, these data indicate that the two reactive Cys residues are likely located close to the sites of ATP binding. Since Pgp in which all Cys residues are eliminated is still able to confer drug resistance, the two MIANS-reactive Cys residues are not essential to Pgp function. Our results suggest that modification of these residues affects one or more steps in ATP hydrolysis.

The emission spectrum of MIANS provides information on the polarity of its local environment. The observation of a 30 nm blue shift for MIANS bound to Pgp suggests that the probe is located in a relatively hydrophobic region within the NBD. The magnitude of the blue shift for the MIANS-

labeled Cys is quite large; in fact, it is comparable to the shift observed for MIANS labeling of several Cys residues within lactose permease helix V, which makes up part of the membrane-spanning domain (Wu & Kaback, 1994). It is possible that this nonpolar environment reflects the existence of a hydrophobic pocket, which might be expected to be present at the active site to accommodate the aromatic rings of the adenine base.

Given the probable close proximity of the bound MIANS to the catalytic site, it is expected that nucleotide binding would affect the fluorescence properties of the probe. This is indeed the case; as shown in Figures 6B and 7, addition of ATP resulted in concentration-dependent quenching of the MIANS fluorescence. The binding of ATP alters the quantum yield of the probe, without changing the emission maximum. Parameters describing ATP binding were extracted after fitting of the fluorescence quenching titration data to an equation describing interaction with a single type of binding site. The resulting K_d value (0.46 mM) was very similar to both the K_M for ATP hydrolysis (0.4 mM) and the IC_{50} value (0.37 mM) for protection against ATPase inactivation by MIANS (Figure 2B).

These findings clearly indicate that ATP is still able to bind to Pgp after reaction with MIANS, and that the affinity of this binding remains unchanged despite covalent modification of Cys 428 and 1071. We have confirmed that MIANS−Pgp retains the ability to bind nucleotides using the fluorescent trinitrophenyl derivative of ATP (TNP-ATP). Addition of this nucleotide probe to MIANS-modified Pgp leads to a large enhancement of TNP-ATP fluorescence, which is concentration-dependent and saturable. In addition, binding of TNP-ATP results in quenching of >80% of the MIANS fluorescence, indicating that the bound nucleotide is located relatively close to the modified Cys (R. Liu and F. J. Sharom, manuscript in preparation). Since covalent modification of the two Cys residues does not affect ATP binding, it seems likely that one or more steps in the catalytic mechanism of ATP hydrolysis itself must be inhibited. This inhibition could arise from the steric effect of introducing a bulky group into the active site region.

It was not possible in this study to determine whether ATP was bound to both NBDs, or only one. Recent work by Senior and co-workers has indicated that only a single mole of nucleotide is trapped by vanadate, resulting in complete inhibition of Pgp ATPase activity (Urbatsch et al., 1995). In light of these results, it seems possible that the MIANS quenching noted in the present study arises from binding of ATP to only one of the two MIANS-labeled NBDs. This may perhaps explain the observation that the quenching induced by binding of a saturating concentration of ATP is less than that induced by saturating concentrations of several drugs (see Figure 6B), despite the fact that the site of ATP binding is presumably much closer to the probe than the site for drug binding (see below).

Addition of various drugs (vinblastine, colchicine, daunorubicin, doxorubicin) and chemosensitizers (verapamil, reserpine, trifluoperazine) also resulted in various degrees of quenching of the MIANS probe. There is considerable evidence to support the concept that the drug binding site of Pgp resides within the membrane-bound regions of the transporter, especially membrane-spanning segments 5, 6, 11, and 12. It has been reported that half-molecules of Pgp showed constitutive ATPase activity, but were no longer stimulated by drugs, whereas coexpression of the two half-molecules restored drug stimulation of ATPase activity (Loo & Clarke, 1994). Since the coupling of drug binding to ATPase activity arises from interactions between the two half-molecules of Pgp, it was suggested that the drug binding site is formed by contributions from residues in each half. In contrast, the NBDs appear to be separately folded soluble protein domains. Indeed, in prokaryotic ABC transporters such as histidine permease and the arsenite transporter, they exist as separate subunits. Therefore, it seems unlikely that the binding site for drug substrates within Pgp is physically close enough to the MIANS probe within the NBDs to alter its fluorescence properties via a "direct" effect. The most likely explanation for the observed quenching by drugs is that it is an "indirect" effect; *i.e.*, drug binding triggers a conformational change within the NBDs, which affects the quantum yield of the bound probe. Two drugs, daunorubicin and trifluoperazine, also produced slight alterations in the polarity of the probe environment, as indicated by a shift in the λ_{max} of emission.

The existence of a long-range conformational change resulting in drug binding was also demonstrated by the fact that the rate of labeling of Pgp with MIANS was substantially reduced in the presence of various drugs and chemosensitizers. The extent of the reduction in labeling rate varied with the particular drug or chemosensitizer under study, but did not appear to correlate with the K_d for binding, as measured by fluorescence quenching. These results suggest that the conformational change which follows drug binding results in reduced accessibility of Cys 428 and Cys 1071 within the NBDs to reaction with MIANS.

Quenching of the MIANS probe by drugs occurs in the absence of ATP, clearly indicating that prior binding of ATP is neither necessary for drug interaction with Pgp nor essential for induction of the conformational change in the NBDs. The extent of quenching induced by saturating concentrations of various drugs and chemosensitizers varied over a wide range, and did not appear to correlate with the affinity of the drug for binding to Pgp. For example, vinblastine, a high-affinity substrate ($K_d = 0.77\ \mu$M), induced a low maximum level of quenching of ~17%, while daunorubicin ($K_d = 10.5\ \mu$M) quenched up to 53% of the MIANS fluorescence (Table 2). The drugs shown in Table 2 have different effects on the ATPase activity of hamster Pgp, both in CHAPS and in a membrane environment. Some compounds substantially increase ATPase activity (*e.g.*, verapamil, trifluoperazine), others stimulate ATPase activity slightly (*e.g.*, colchicine), and some cause inhibition of activity (*e.g.*, vinblastine, doxorubicin, daunorubicin) (Sharom et al., 1993, 1995a). Yet all these drugs induce quenching, suggesting that they all give rise to a putative conformational change in the NBD which can be detected by the MIANS probe. Presumably, the change produced by some drugs leads to enhanced catalytic activity, whereas the change produced by others has the opposite effect. It is still not clear whether drugs and/or chemosensitizers share the same binding site within the Pgp molecule. Since all the compounds tested produced quenching to various degrees, it is possible that they interact with different overlapping regions of a large drug binding site.

The maximum degree of quenching, $\%(\Delta F_{max}/F_0)$, appears unrelated to the magnitude or direction of the effect of the drug on the ATPase activity (Table 2). For example, the

maximum degree of quenching observed for verapamil is one of the smallest, yet this chemosensitizer produces a large stimulation in ATPase activity (Sharom et al., 1993, 1995a). In contrast, the largest quenching effect was noted for colchicine and daunorubicin, yet the former gives only a small increase in ATPase activity, and the latter is inhibitory (Sharom et al., 1993, 1995a). The large degree of quenching observed for these two drugs suggests that they induce a substantial change in the environment of the MIANS probe. All of the drugs listed in Table 2 compete for drug transport by Pgp, and all are likely transported by Pgp.

Experiments in which Pgp was titrated first with ATP and then with vinblastine or verapamil indicated that the quenching effects of nucleotide and drug were independent and additive. This indicates that binding of ATP and drug gives rise to separate, independent changes in the Pgp molecule. Verapamil quenching was essentially unaltered by previous binding of ATP, whereas a reduction in the maximal extent of quenching was noted for vinblastine. However, the affinity of drug interaction with Pgp was not affected by prior binding of ATP, suggesting that ordered substrate binding to the multidrug transporter is not a feature of its mechanism of action.

Both the ATPase activity and transport function of Pgp are highly dependent on the presence of phospholipids (Doige et al., 1993; Sharom et al., 1995a; Urbatsch & Senior, 1994; Senior et al., 1995). Detergent-solubilized Pgp often displays substantially lower levels of ATPase stimulation by drugs than membrane-bound Pgp, and it has been suggested that detergent may unfold the protein somewhat, thus reducing the coupling between the drug binding site(s) and the catalytic domains (Sharom et al., 1995a). Asolectin (soybean phospholipids) is a natural, very fluid lipid mixture that was previously shown to be highly effective in both protecting Pgp ATPase activity from thermal inactivation and restoring ATPase activity following detergent delipidation (Doige et al., 1993). In the absence of phospholipids, the changes in fluorescence intensity observed following addition of ATP and drugs to MIANS−Pgp were smaller and variable. Addition of phospholipids thus appears to assist Pgp to refold into a more native conformation, in which coupling between the drug binding site and the NBD is restored.

This is the first report of measurement of parameters for nucleotide and drug binding to Pgp. It is clear that fluorescence quenching techniques should enable the exploration of factors that may modulate drug binding, such as the nature of the lipid environment, Pgp phosphorylation, etc. In addition, it is now possible to use stopped-flow fluorescence to measure the kinetic constants, such as on and off rates, for interaction of various drugs and nucleotides with purified Pgp.

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