# Characterization of the ATPase Activity of Purified Chinese Hamster P-glycoprotein

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ABSTRACT: A simple and rapid procedure is described for purification of P-glycoprotein (Pgp) from a multidrug-resistant Chinese hamster ovary cell line (CR1R12) in which the plasma membranes are highly enriched in Pgp (Al-Shawi, M. K., Senior A. E. (1993) *J. Biol. Chem. 268*, 4197–4206). The procedure consisted of octylglucoside solubilization of Pgp from plasma membranes and chromatography on Reactive Red 120 agarose. The purified Pgp displayed substantial verapamil-stimulated MgATPase activity ($k_{cat}$ = 9.2 $s^{-1}$, $K_M$(MgATP) = 0.8 mM). A range of other compounds known to interact with Pgp in whole cells also stimulated the MgATPase activity. Catalytic activity in presence of verapamil was characterized in terms of pH dependence, magnesium versus calcium specificity, kinetic parameters, nucleotide specificity, and inhibitors. There was potent inactivation of MgATPase activity by NEM and NBD-Cl, which was diminished greatly by MgATP protection. Vanadate was also an effective inhibitor. Predominantly, the catalytic features seen resembled those reported previously for the plasma membrane-bound form of Pgp. The catalytic nucleotide-binding sites are therefore preserved in their native folded conformation in the purified Pgp preparation.

P-glycoprotein (Pgp),[1] also known as multidrug-resistance protein, is a plasma-membrane protein which is responsible for ATP-dependent exclusion of multiple drugs from cells (Riordan & Ling, 1985; Endicott & Ling, 1989; Gottesman & Pastan, 1993). The amino acid sequences of human, mouse, rat, and Chinese hamster Pgp isoforms are very similar and around 1280 amino acid residues in length. They exhibit strong homologies to nucleotide-binding proteins and to a large ("ABC") superfamily of membrane-associated transport proteins (Juranka *et al.*, 1989; Ames *et al.*, 1990; Higgins, 1992).

The detailed mechanism by which Pgp acts to exclude drugs (and a variety of other compounds) from cells is currently unknown, but there is intense interest in this topic because of its relevance to cancer chemotherapy (Roninson, 1992; Gottesman, 1993; Goldstein *et al.*, 1992). Studies with plasma-membrane vesicles (Horio *et al.*, 1988, 1991; Lelong *et al.*, 1992; Doige & Sharom, 1992) or with reconstituted proteoliposomes containing partially-purified Pgp (Sharom *et al.*, 1993) have demonstrated that nucleoside triphosphate hydrolysis is required for drug uptake and that nucleotide binding is not sufficient.

Substantial drug-stimulated ATPase activity, comparable to that of other transport ATPases, has been reported for Chinese hamster and human Pgp in preparations of plasma membranes (Sarkadi *et al.*, 1992; Al-Shawi & Senior, 1993), detergent-extracted Pgp (Doige *et al.*, 1992), or partially-purified and reconstituted Pgp (Ambudkar *et al.*, 1992; Sharom *et al.*, 1993). Detailed characterization of the structure and function of the Pgp catalytic sites is an important goal which we are pursuing in our laboratory. While it is important to establish the properties of Pgp in plasma membranes (e.g. Al-Shawi *et al.*, 1994), incisive biochemical studies will clearly require a highly-purified Pgp preparation. Also, in the longer term, it is to be anticipated that structure resolution by X-ray crystallography will be possible, and this will also depend upon a purification method for P-glycoprotein being available.

Here we report a simple and rapid procedure for purification of Pgp. We described previously a highly multidrug-resistant Chinese hamster ovary cell-line (CR1R12) that constitutively overexpresses Pgp. Plasma membranes from CR1R12 cells are considerably enriched in Pgp (Al-Shawi & Senior, 1993) and provided an excellent source material for our purification scheme. We further report here a detailed characterization of the MgATPase activity and other catalytic properties of the purified Pgp preparation.

## MATERIALS AND METHODS

*Plasma Membrane Preparation.* Chinese hamster ovary CR1R12 cells were grown in suspension culture and plasma membranes were prepared from the harvested cells as described previously (Al-Shawi & Senior, 1993). The final step of preparation involved discontinuous sucrose gradient centrifugation, and the plasma membranes accumulated at the 16/31 sucrose interface (i.e., 16% and 31% w/v sucrose). They contained 15–20% (w/w) Pgp as a percent of total membrane protein, estimated as described in Al-Shawi *et al.* (1994). There was also a membranous fraction which accumulated at the 31/45 sucrose interface, referred to in this paper as "31/45 membranes", which is a heterogeneous population of intracellular membranes and plasma membranes (Riordan & Ling, 1979). The "31/45 membranes" were less enriched in Pgp (3–5%, w/w, as percent of total membrane protein), but as we describe in Results, this material also provided a valuable source of purified Pgp.

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[1] Abbreviations: Pgp, P-glycoprotein; OG, *n*-octyl β-D-glucopyranoside; DCCD, dicyclohexylcarbodiimide; FITC, fluorescein isothiocyanate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; *lin*-benzo-ATP, 8-amino-3-(β-D-ribofuranosyl)imidazo[4,5-*g*]-quinazoline 5′-triphosphate (it has formally a benzene ring inserted between pyrimidine and imidazole rings of adenine, linearly extending the ring system); AMP-PNP, adenyl-5′-yl imidodiphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); $TPP^+$, tetraphenylphosphonium; $TPA^+$, tetraphenylarsonium; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; DTT, dithiothreitol.

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*Assays of ATPase Activity.* For membrane-bound and detergent-solubilized Pgp, reactions were carried out in 50 μL of 40 mM Tris-Cl, pH 7.4, 0.1 mM EGTA, 2 mM ouabain, 10 μM verapamil, 10 mM $MgSO_4$, and 10 mM NaATP at 37 °C for appropriate times during which the reaction was shown to be linear and ≤10% of added nucleotide was hydrolyzed. For purified Pgp, reactions were carried out as above, except that EGTA and ouabain were not required and verapamil concentration was 50 μM. Reactions were started by addition of Pgp or substrate (MgATP or Mg-nucleotide) and stopped by addition of 1 mL of 20 mM ice-cold $H_2SO_4$. $P_i$ released was assayed by the method of Van Veldhoven and Mannaerts (1987). For determination of kinetic parameters, nucleotide and divalent cation concentrations were adjusted as required. Concentrations of ionic species of magnesium, calcium, and ATP were calculated according to Fabiato and Fabiato (1979). Solutions of activators or inhibitors were added in dimethyl sulfoxide, except that NBD-Cl was added in ethanol and the final concentration of the solvent was ≤1% (v/v) in the assays. pH dependence of ATPase activity of purified Pgp was measured as in Al-Shawi and Senior (1993), except that verapamil concentration was 50 μM.

*Reconstitution of Solubilized Pgp in Proteoliposomes.* Solubilized Pgp preparations, which contained octyl glucoside (OG) and *Escherichia coli* lipid as described later, were dialyzed for 16 h at 0 °C against 20 volumes of buffer containing 50 mM Tris Cl, pH 7.4, 1 mM DTT, 1 mM EGTA, and 5 mM 6-aminohexanoic acid, with one buffer change at 3 h. The initial lipid:protein ratio was 130:1 by weight. The proteoliposomes were harvested by centrifugation (250000*g* × 60 min) and resuspended in the same buffer at a protein concentration of 0.5–1.0 mg/mL. The recovery of total protein and lipid in the pellet was around 70%. The proteoliposomes were frozen in aliquots at −70 °C and exposed to a single thaw cycle before each experiment.

*Routine Procedures.* SDS gel electrophoresis, immunoblotting, and protein assays by bicinchoninic acid method in presence of 1% SDS were all performed as previously described in Al-Shawi and Senior (1993). For proteoliposomes or fractions containing lipids, the samples were first delipidated by the method of Wessel and Flügge (1983). Silver staining of SDS gels utilized a prereduction step as described by Heukeshoven and Dernick (1988); the gels were preincubated in water containing 0.325 mM DTT for 1 h at room temperature before exposure to $AgNO_3$ solution (S. V. Ambudkar, personal communication). Laser densitometry of silver-stained SDS gels showed that in the range of 0.1–1.5 μg of Pgp applied, the integrated staining intensity was linear and was the same as for bovine serum albumen.

*Materials.* Tissue culture materials were from GIBCO. Dye-ligand chromatography reagents Reactive Red 120 agarose Type 3000-Cl (Cat. No. R0503), Cibacron Blue Type 3000-Cl (Cat. No. C1535), and routine chemicals were from Sigma. Octylglucoside (OG, *n*-octyl β-D-glucopyranoside) was from Calbiochem. *E. coli* lipids were obtained from Avanti Polar Lipids; L-α-phosphatidylcholine from egg yolk and soybean were from Sigma. Monoclonal C219 antibody was from Centocor Diagnostics and monoclonal C494 antibody was from Signet Laboratories.

## RESULTS

*Solubilization of P-glycoprotein from Plasma Membranes of Chinese Hamster Ovary CR1R12 Cells.* In preliminary experiments several ionic, nonionic, and zwitterionic detergents were tested, and it was found that octyl glucoside (OG) was among the most effective in solubilizing Pgp from the CR1R12 plasma membranes. In a typical experiment, OG at 1% (w/v) concentration, in the presence of osmolyte glycerol (20% v/v) (Maloney & Ambudkar, 1989), gave ≥90% solubilization of Pgp. The total ATPase activity of OG-solubilized Pgp was considerably (~80%) inhibited, probably due to removal of tightly-associated lipid by the detergent (Doige *et al.*, 1992; 1993). The activity could be restored fully by addition of lipid, for example L-α-phosphatidylcholine from egg yolk or soybean, crude *E. coli* lipid, or acetone/ether precipitated *E. coli* lipid. The ATPase activity (expressed as units/mg protein) of OG-solubilized supernatant from plasma membranes, supplemented with 1% (w/v) acetone/ether precipitated *E. coli* lipid, was 1.5-fold higher than that of plasma membranes, and partial enrichment of Pgp in the OG-solubilized supernatant was apparent from SDS gels. Verapamil stimulated the ATPase activity of OG-solubilized, lipid-supplemented Pgp maximally by 2.5-fold and the optimal verapamil concentration was 50–100 μM. Further experiments showed that OG-solubilized Pgp could be reconstituted directly into proteoliposomes (following the procedure described in Methods) with full retention of verapamil-stimulated ATPase and with further enrichment of Pgp in proteoliposomes.

OG was therefore chosen as detergent for solubilization of Pgp. The standard solubilization procedure adopted was to incubate plasma membranes at 1–1.5 mg of protein/mL in buffer containing 50 mM Tris Cl, pH 7.0, 6.5 mM DTT, 0.25 M sucrose, 1 mM EGTA, 5 mM 6-aminohexanoic acid, 1 mM $NaN_3$, 1 μM leupeptin, 2 μM pepstatin A, 20% v/v glycerol, 1.4% w/v OG, 0.4% w/v acetone/ether precipitated *E. coli* lipid, and 50 mM MgATP for 15 min at 4 °C; this resulted in solubilization of 60–70% of total membrane protein and ≥90% of Pgp, which remained in the supernatant after centrifugation at 150000*g* for 60 min.

*Purification of Solubilized Pgp from Plasma Membranes on Reactive Red 120.* The Reactive Red 120 column (1 mL packed resin per 2 mg of solubilized protein) was preequilibrated with five bed volumes of buffer containing 50 mM Tris-Cl, pH 7.0, 6.5 mM DTT, 0.25 M sucrose, 1 mM EGTA, 5 mM 6-aminohexanoic acid, 1 μM leupeptin, 2 μM pepstatin A, 20% v/v glycerol, 1.25% w/v OG, 0.2% w/v acetone/ether precipitated *E. coli* lipid, 50 mM $MgCl_2$, and 50 mM NaATP at 4 °C. The supernatant obtained from OG-solubilization of the plasma membranes was applied, and the column was washed with five volumes of buffer. The material that did not adsorb to the column contained contaminating proteins and some Pgp. The column was then eluted with a linear gradient of 0–500 mM NaCl in buffer (total volume was five times column volume). Purified Pgp eluted in a broad peak between 50 and 450 mM NaCl. The column fractions eluting at 50–450 mM NaCl were pooled and the Pgp was reconstituted into proteoliposomes as described in Methods. The material remaining on the column after the NaCl gradient was eluted with 5% SDS and was seen to contain mostly contaminating proteins and a small amount of Pgp. Figure 1 is an SDS gel which documents the progress of the purification scheme. It demonstrates that the pooled column fractions eluting from Reactive Red 120 with 50–450 mM NaCl, after reconstitution in proteoliposomes, contained Pgp that was highly purified (lanes 5 and 7) and that the purified Pgp cross-reacted with monoclonal C219 and polyclonal anti-Pgp antibodies (lanes 10 and 11).

The choice of resin to be used for purification was empirical. It would appear that ionic interactions rather than an affinity

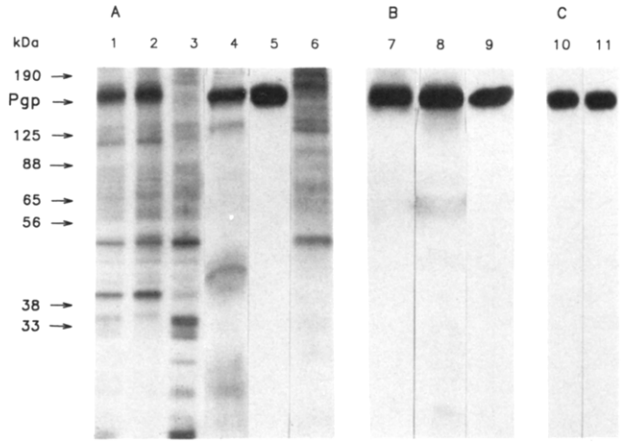


FIGURE 1: SDS gel electrophoresis and immunoblotting of P-glycoprotein at various stages of purification. (A) Coomassie blue-stained SDS gel. Lane 1, plasma membranes (10 μg); lane 2, supernatant from OG extract of plasma membranes (10 μg); lane 3, pellet from OG extract of plasma membranes (10 μg); lane 4, pooled fractions eluted from Reactive Red 120 column with 0–50 mM NaCl (10 μg); lane 5, pooled fractions eluted from Reactive Red 120 column with 50–450 mM NaCl after reconstitution in proteoliposomes as described in Methods (5 μg); lane 6, fraction eluted from Reactive Red 120 column with 5% SDS (10 μg). (B) Silver-stained SDS gel. Lane 7, pooled fractions eluted from Reactive Red 120 column with 50–450 mM NaCl after reconstitution in proteoliposomes (1 μg) (same as lane 5 above); lane 8, "31/45 membranes" (see Methods) were used as starting material for OG solubilization and the pooled fractions eluted from the Reactive Red 120 column with 50–450 mM NaCl were reconstituted in proteoliposomes (1.5 μg); lane 9, the material in lane 8 was further purified on a Cibacron Blue column. Protein was eluted from the Cibacron Blue column with 1 M NaCl and reconstituted in proteoliposomes (1 μg). (C) Immunoblotting of the purified P-glycoprotein. The purified P-glycoprotein obtained by OG solubilization of plasma membranes and Reactive Red 120 chromatography (as shown in lane 5 and lane 7) was immunoblotted with monoclonal C219 antibody (lane 10) or polyclonal anti-P-glycoprotein antibodies (Al-Shawi & Senior, 1993) (lane 11). Similar results were seen when purified Pgp obtained from "31/45 membranes" (as in lane 9) was used (data not shown).

for the Pgp nucleotide binding fold(s) caused adsorption of the Pgp to the Reactive Red 120.

*Further Purification of Solubilized Pgp from "31/45 membranes" on Cibacron Blue.* When "31/45 membranes" (described in Methods) were used as starting material instead of plasma membranes, minor contaminants were present in the pooled fractions obtained after elution from Reactive Red 120 with buffer containing 50–450 mM NaCl (Figure 1, lane 8). This was no doubt due to the fact that the "31/45 membranes" were less enriched in Pgp than the plasma-membrane preparation, by approximately 5-fold. A Cibacron Blue column (2 mL of packed resin per mg of protein, preequilibrated in the same buffer as for the Reactive Red 120 column) was used for further purification. The pooled fractions obtained after elution from Reactive Red 120 with buffer containing 50–450 mM NaCl were applied directly to the Cibacron Blue, the column was washed with five volumes of buffer, and then the column was eluted with buffer containing 1 M NaCl. The material eluted with 1 M NaCl was reconstituted into proteoliposomes as described in Methods. On SDS gels these proteoliposomes were seen to contain highly purified Pgp (Figure 1, lane 9).

*Yield of Purified Pgp.* The yield of purified Pgp was typically 250 μg from 2.5 mg of plasma membranes or 400 μg from 22 mg of "31/45 membranes". The protein amounts given are those obtained typically from one batch of cells (8 × 1.25 L of suspension culture). It can be seen therefore that both the plasma membrane preparation and the "31/45 membranes" were good sources of purified Pgp.

*Purified P-glycoprotein Isoform.* Immunoblots showed that the purified Pgp cross-reacted strongly with both C494 and C219 monoclonal antibodies. Since C494 is specific for Pgp isoform-1 whereas C219 cross-reacts with all three isoforms (Georges *et al.*, 1990), this indicated that isoform-1 was at least a major component. Immunoprecipitation experiments then showed that the majority of the purified Pgp after solubilization was precipitated by the C494 antibody and that the protein remaining in the supernatants was cross-reactive with both C494 and C219 antibodies (data not shown). From these experiments we concluded that Pgp isoform-1 is the predominant form (>95%) in the purified Pgp preparation, although we cannot rule out the presence of minor amounts of other isoforms. Previous work had shown that Pgp isoform-1 is the predominant isoform in plasma membranes from CR1R12 cells (Al-Shawi *et al.*, 1994). Chinese hamster P-glycoprotein isoform-1 is analogous to human multidrug-resistance protein isoform-1 (*mdr1*), which is responsible for multidrug resistance in cancer (Gottesman and Pastan, 1993).

*Characterization of the Catalytic Activity of Purified Pgp.* All of the experiments described below were performed on purified Pgp obtained from plasma membranes after OG solubilization, Reactive Red 120 column chromatography, and reconstitution in proteoliposomes, as described above. We confirmed that the purified Pgp obtained using "31/45 membranes" as the starting material gave the same results.

(1) *ATPase Activity.* The specific ATPase activity in the absence of verapamil was 2.1 ± 0.3 (S.D.) μmol/min/mg Pgp. Variation of the osmolarity of the assay medium by inclusion of sucrose at different concentrations, or addition of low concentrations of detergents, did not increase the specific ATPase activity, demonstrating that access of substrate to Pgp catalytic sites was not restricted. Verapamil gave maximally 1.5- to 2.6-fold stimulation in eight different preparations and the concentration of verapamil required for maximal stimulation was 50–100 μM. The following average kinetic parameters for MgATPase activity were determined from multiple experiments: $K_M$(MgATP) (no verapamil) = 0.8 mM; $k_{cat}$ = 4.9 $s^{-1}$ (a molecular mass of 141 000 Da for Pgp was used); $K_M$(MgATP) (with 50 μM verapamil) = 0.8 mM; $k_{cat}$ = 9.2 $s^{-1}$; $k_{cat}/K_M = 1.2 \times 10^4$ $M^{-1}$ $s^{-1}$. The ATPase activity of purified Pgp in proteoliposomes was fully stable at −70 °C for at least 3 months. Also the activity remained stable at 4 °C for several weeks. There was zero detectable Na,K-ATPase or Ca-ATPase activity (assayed ±2 mM ouabain and ±1 mM EGTA, respectively) nor any ecto-ATPase activity (assayed as described by Lin and Guidotti, 1989).

Shapiro and Ling (1994) recently noted that 10 μM verapamil elicited a 5.4-fold increase in apparent $K_M$(ATP) for ATPase activity in Chinese hamster P-glycoprotein reconstituted in crude brain lipids, in contrast to what was seen here. One speculation could be that the lipid environment may influence the kinetic properties seen in response to the drug (see Discussion).

(2) *pH Dependence of ATPase Activity.* The pH dependence of ATPase activity of purified Pgp (Figure 2) showed a broad range of activity with an optimum at around pH 7.5, in presence or absence of verapamil.

(3) *Magnesium versus Calcium Specificity.* From Figure 3A it may be seen that magnesium was a far better activator of verapamil-stimulated ATPase activity than calcium. There

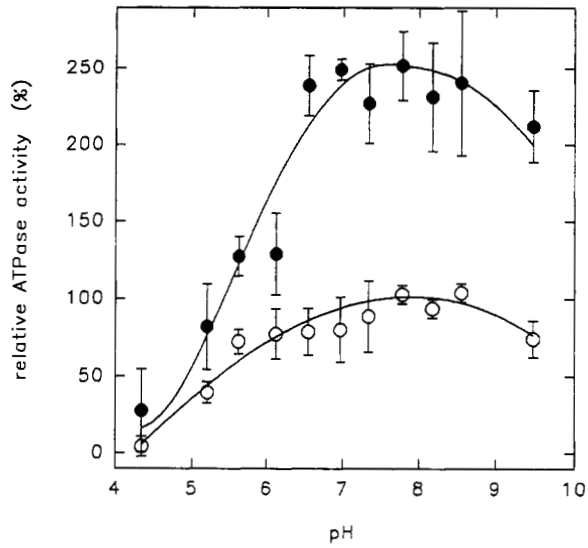


FIGURE 2: pH dependence of the ATPase activity of purified P-glycoprotein. ATPase activity was measured as described in Methods. Results are averages of quadruplicate determination ± std dev (bars). (●) with 50 μM verapamil; (○) no verapamil.

was apparent activation by magnesium at concentrations up to 25 mM, and because the maximal concentration of MgATP was 10mM in these experiments, this implied that there was a small activating effect of free $Mg^{2+}$. Figure 3B shows enzyme activity as a function of cation-ATP complex concentrations and emphasizes the superiority of MgATP as substrate. In the absence of divalent cation and presence of 0.1 mM EDTA, there was no measurable hydrolysis of 10 mM NaATP.

(4) *Nucleotide Specificity*. Table 1 shows that a range of nucleoside triphosphates served as substrates for hydrolysis by purified Pgp in the presence of verapamil, and the kinetic parameters obtained showed that the catalytic sites are somewhat nonspecific. For example, ATP analogs in which the base moiety is extended by substitution at the 8- or 1,$N^6$-positions (as in 8-bromo-, 8-azido-, and 1,$N^6$-etheno-ATP), or by insertion of an extra ring system (as in *lin*-benzo-ATP), were apparently good or at least reasonably good substrates. Removal of the 2′-hydroxyl from the ribose moiety had little effect on activity. 8-Azido- and 8-bromo-ATP normally assume predominantly the *syn* conformation in solution as opposed to the *anti* conformation for ATP (Czarnecki, 1984) but this also had little apparent effect. GTP, ITP, UTP, and CTP were poor but real substrates for hydrolysis. ADP, AMPPNP, and ATPγS were all found to be classical competitive inhibitors of MgATPase activity (Table 1) and none of these was hydrolyzed by the purified Pgp. Compared to their inhibitory potencies in other systems, both AMPPNP and ATPγS seemed to inhibit purified Pgp relatively weakly, supporting the view advanced earlier that Pgp may not contain a high affinity binding site for ATP (Al-Shawi *et al.*, 1994). AMP was neither a substrate nor an inhibitor. Two of the nucleotides shown to be substrates in Table 1 (namely *lin*-benzo-ATP and 1,$N^6$-etheno-ATP) are fluorescent analogs of ATP which could prove valuable in future studies, and 8-azido-ATP is of course a potential photoaffinity labeling reagent for the catalytic sites.

(5) *Activators of Pgp ATPase Activity*. A representative selection chosen from the large number of compounds which have been shown in the literature to interact with Pgp in whole cells was tested for ability to stimulate the ATPase activity of purified Pgp. Figures 4A and 4B show titration experiments with eight of these compounds. Table 2 gives measured values

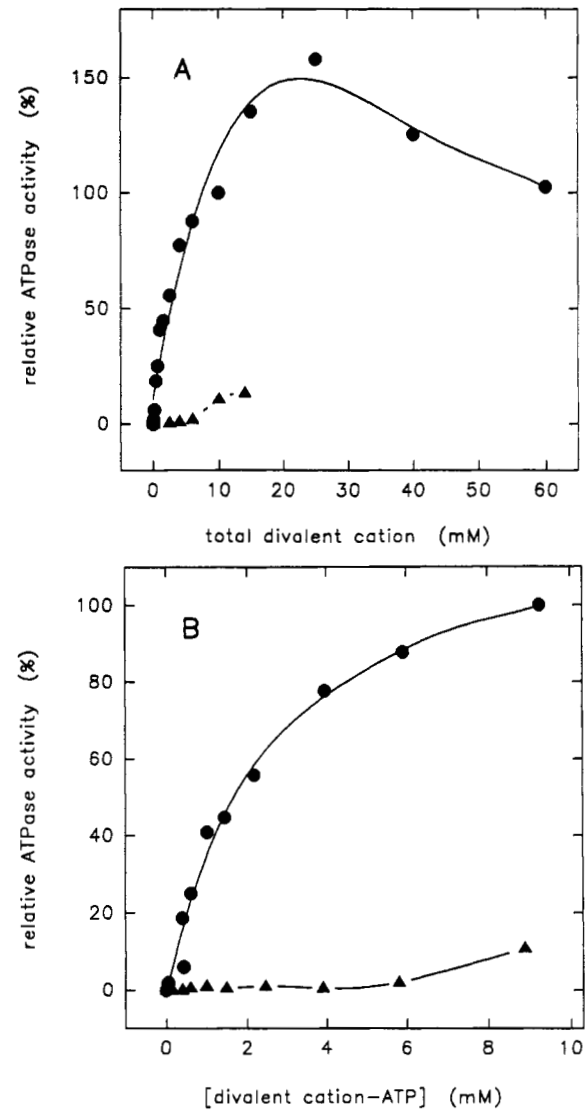


FIGURE 3: Magnesium and calcium dependence of the ATPase activity of purified P-glycoprotein. ATPase activity was measured at 37 °C in 10 mM NaATP, 40 mM Tris-Cl, pH 7.4, 50 μM verapamil, with 0.05–60 mM $MgSO_4$ or 0.05–14 mM $CaSO_4$. (A) Relative ATPase activity as a function of total divalent cation added. 100% is defined as the activity at 10 mM added $MgSO_4$. (B) Relative ATPase activity as a function of divalent cation-ATP complex concentration; 100% was defined as the same value as in A.

of the concentration required for half-maximal activation, and the maximal activation seen, for a total of 15 compounds which were seen to give stimulation. Not all of the compounds tested were found to stimulate ATPase activity; notably colchicine, vinblastine, vincristine, reserpine, adriamycin, and daunomycin did not stimulate. This was somewhat unexpected since (with the exception of reserpine which has not been studied) each of these compounds has been shown previously to be a transport substrate for Pgp in intact CR1R12 cells, either by direct uptake studies or by assays of drug resistance (Al-Shawi & Senior, 1993; Gibson *et al.*, submitted for publication). It may be noted that a similar phenomenon has recently been reported by Sharom *et al.* (1993). These workers selectively extracted Pgp from Chinese hamster ovary cells using the detergent CHAPS and reconstituted it in proteoliposomes. The resultant partially (~50%) purified preparation of Pgp showed up to 2-fold stimulation of ATPase activity by verapamil and trifluoperazine, at concentrations similar to those seen to stimulate here (Figure 4A). Remarkably, colchicine, vinblastine, and daunomycin did not significantly

Table 1: Specificity of Purified P-glycoprotein for Mg-nucleotides as Substrates and Inhibitors[a]

| nucleotide | $V_{max}$ (μmol/mg Pgp/min) | $K_M$ (mM) | $k_{cat}/K_M$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|
| ATP | 3.9 | 0.8 | $1.2 \times 10^4$ |
| 2′-dATP | 2.0 | 1.1 | $4.3 \times 10^3$ |
| 8-bromo-ATP | 1.1 | 1.3 | $2.0 \times 10^3$ |
| 8-azido-ATP | 1.2 | 0.5 | $5.6 \times 10^3$ |
| 1,$N^6$-etheno-ATP | 2.7 | 2.0 | $3.2 \times 10^3$ |
| *lin*-benzo-ATP[b] | 0.90 | N.D. | |
| GTP[c] | 0.14 | N.D. | |
| ITP[c] | 0.21 | N.D. | |
| UTP[b] | 0.06 | N.D. | |
| CTP[b] | 0.15 | N.D. | |
| ADP | 0[d] | $K_i = 0.70$ | |
| AMP-PNP | 0[d] | $K_i = 0.35$ | |
| ATPγS | 0[d] | $K_i = 0.07$ | |
| AMP | 0[d] | $K_i > 10.0$ | |

[a] Assays were performed at 37 °C, in 40 mM Tris-Cl, pH 7.4, 50 μM verapamil, with varied concentration of Mg-nucleotide for $V_{max}$ and $K_M$ determination, or with varied MgATP and inhibitor concentrations for $K_i$ determination. For further details, see Methods. [b] $V_{max}$ determined at 10 mM Mg-nucleotide, $K_M$ not determined. [c] The $K_M$ values for GTP and ITP were high (>3 mM) and could not be calculated accurately. The $V_{max}$ values were obtained at 10 mM Mg-nucleotide and may also be underestimated. [d] No detectable activity.

stimulate ATPase activity, even though there was good evidence that colchicine was actually transported by Pgp in the same proteoliposomes and that vinblastine and daunomycin competed effectively with colchicine transport (Sharom *et al.*, 1993). Possible explanations for these findings will be discussed later.

(6) *Inhibitors of Verapamil-Stimulated ATPase Activity.* Inhibition by orthovanadate was investigated and the results are shown in Figure 5. The concentration required for half-maximal inhibition was 9 μM (Table 3), which is very similar to the corresponding value of 12 μM found previously for Pgp in CR1R12 cell plasma membranes by Al-Shawi and Senior (1993). This is much higher than the concentrations required to inhibit "P-type" ATPases which utilize a covalent phosphorylated reaction intermediate ($K_i$ values = 50–500 nM, Cantley *et al.*, 1978; O'Neal *et al.*, 1979; Inesi, 1985) but supports the idea that a pentacoordinate phosphorus species occurs during catalysis. DCCD was a weak inhibitor (Table 3), implying that carboxyl groups are not critical for activity. The very weak inhibition by FITC and lack of inhibition by azide serve very effectively to discriminate Pgp ATPase activity from that of Na/K-ATPase and mitochondrial ATPase, respectively.

ATPase activity of Pgp in plasma membranes is potently inactivated by both NBD-Cl and NEM. The former reagent appears to inactivate by reacting in an ATP-protectable manner with a single group in the enzyme, probably lysine, and the latter reagent reacted in an ATP-protectable manner with two groups in the enzyme, probably cysteines (Al-Shawi & Senior, 1993; Al-Shawi *et al.*, 1994). Therefore, it was important to test whether purified Pgp was also inactivated by these reagents.

The inactivation of purified Pgp by NEM is shown in Figure 6. For comparison we have included data showing the inactivation of plasma-membrane-bound Pgp by NEM, and one can see that both forms of Pgp are inactivated to similar extent and in an ATP-protectable manner. The concentrations of NEM required for half-maximal inactivation were 2 and 5 μM for plasma membranes and purified Pgp, respectively, in these experiments. The inactivation of purified Pgp by

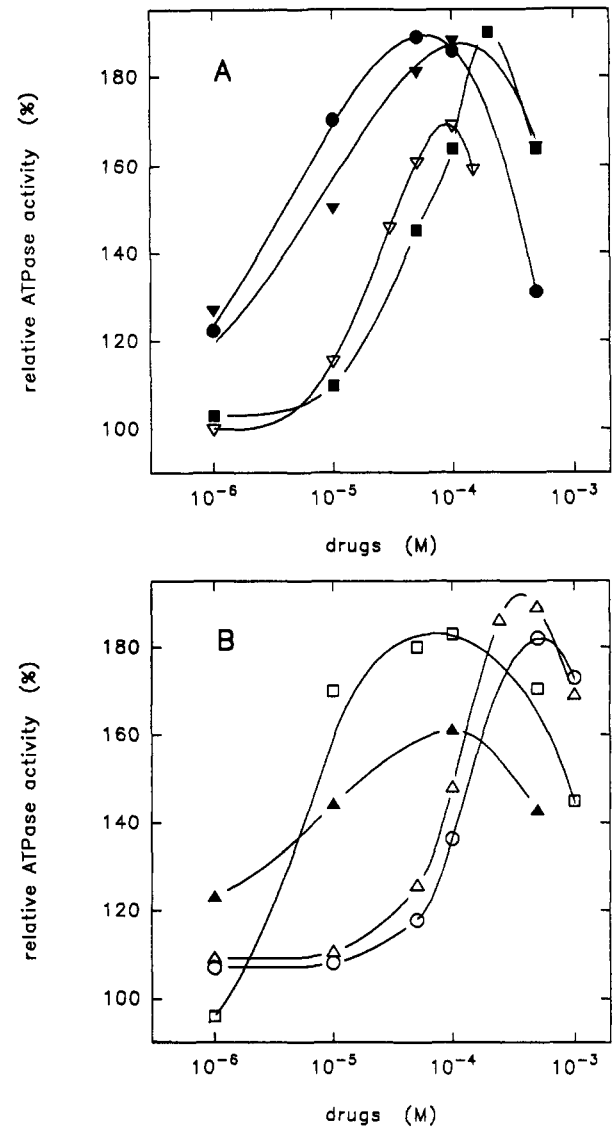


FIGURE 4: Effects of activators on the ATPase activity of purified P-glycoprotein. ATPase activities were assayed at 37 °C in 10 mM NaATP, 40 mM Tris-Cl, pH 7.4, 0.25 M sucrose, 15 mM $MgSO_4$, 0.1 mM EGTA, at a final concentration of Pgp of 3 μg protein per mL; 100% activity was 2.1 μmol of ATP hydrolyzed/min/mg protein. (A) (●) verapamil; (▼) rhodamine-123; (■) trifluoperazine; (▽) fura-2AM. (B) (□) $TPP^+$; (▲) quinidine; (△) pepstatin A; (○) progesterone.

NBD-Cl is shown in Figure 7 and is compared also with the pattern for plasma-membrane-bound Pgp. Here it is seen that while both forms of Pgp were inactivated by NBD-Cl in an ATP-protectable manner, the concentration of NBD-Cl required for 50% inactivation was approximately 4-fold higher in the purified Pgp (20 μM) as compared to plasma-membrane-bound Pgp (5 μM). This may to some degree be due to partitioning of the NBD-Cl into the proteoliposome lipids. The calculated concentrations of NEM and NBD-Cl required for 50% inactivation of purified Pgp in presence of 10 mM MgATP are included in Table 3.

(7) *Stimulation by High Salt Concentrations.* We observed a stimulatory effect of salts on ATPase activity of purified Pgp. Maximal enhancement of activity, in the absence of verapamil, was ~2.5-fold and occurred at 800 mM NaCl or 600 mM $Na_2SO_4$. In the presence of verapamil (50 μM), additional stimulation by salts was insignificant. A possible explanation for this effect is that the high concentration of salt alters the fluidity of the lipid environment of the membrane-embedded portion of the Pgp molecules.

Table 2: Activation of ATPase Activity of Purified P-glycoprotein[a]

| class | compound | $EC_{50}$ ($\mu$M) | maximal activtn (-fold) |
|---|---|---|---|
| Ca channel blocker | verapamil | 3 | 1.5–2.6[b] |
| | nifedipine | 5 | 1.4 |
| calmodulin antagonist | trifluoperazine | 50 | 1.9 |
| fluorescent probe | rhodamine-123 | 5 | 1.9 |
| | fura-2AM[c] | 20 | 1.7 |
| | indo-1AM[c] | 30 | 1.7 |
| steroid hormone | progesterone | 110 | 1.8 |
| | dexamethasone | 200 | 1.4 |
| | hydrocortisone | 300 | 1.6 |
| peptide | ALLN | 300 | 1.5 |
| | leupeptin | 200 | 1.7 |
| | pepstatin A | 90 | 1.9 |
| antiarrhythmic | quinidine | 3 | 1.6 |
| lipophilic cation | $TPP^+$ | 5 | 1.8 |
| | $TPA^+$ | 4 | 1.8 |

[a] ATPase assays were carried out at 37 °C in 40 mM Tris-Cl, pH 7.4, 0.25 M sucrose, 0.1 mM EGTA, 10 mM NaATP, 15 mM $MgSO_4$. $EC_{50}$ is the concentration required to give 50% of maximal activation. Values given are means of at least triplicate estimations, except for verapamil (see footnote *b*). [b] Range of stimulation seen in eight different preparations. [c] The analogous free acid did not stimulate.

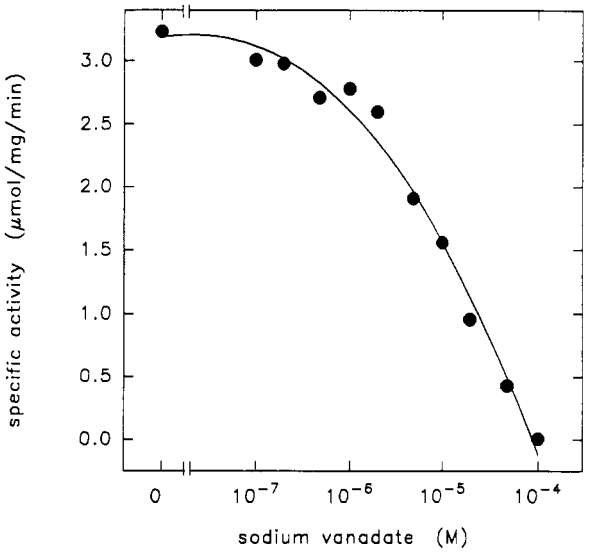


FIGURE 5: Inhibition of the ATPase activity of purified P-glycoprotein by orthovanadate. ATPase activity was assayed at 37 °C in 10 mM MgATP, 40 mM Tris-Cl, pH 7.4, 0.25 M sucrose, 0.1 mM EGTA, 50 $\mu$M verapamil at a final concentration of Pgp of 3 $\mu$g protein per mL with 0.1–100 $\mu$M sodium orthovanadate.

## DISCUSSION

The first goal of this work was to purify Chinese hamster P-glycoprotein, and a simple and rapid procedure was devised for this purpose. Plasma membranes from the highly multidrug-resistant Chinese hamster ovary cell-line CR1R12 were used as source material. The procedure involved solubilization of Pgp with octyl glucoside (OG) in the presence of glycerol and *E. coli* lipid and chromatography on Reactive Red 120. The purified Pgp was reconstituted into proteoliposomes by dialysis. We also show that another membranous fraction obtained from the CR1R12 cells, which we call "31/45 membranes" because it accumulated at the 31%/45% sucrose interface during discontinuous gradient centrifugation, also served as source of purified Pgp, although an additional step of purification on Cibacron Blue was required in this case. The yield of purified Pgp was greatly increased by using both membrane fractions from each batch of cells grown in suspension culture.

Table 3: Inhibitors of the ATPase Activity of Purified P-glycoprotein[a]

| inhibitor | concentration for 50% inhibition ($\mu$M) |
|---|---|
| $Na_3VO_4$[b] | 9 |
| $NaN_3$ | $>5 \times 10^3$ |
| DCCD[b] | >500 |
| FITC[b] | $>1 \times 10^3$ |
| NEM[c] | 5 |
| NEM plus 10 mM MgATP[c] | 600 |
| NBD-Cl[d] | 20 |
| NBD-Cl plus 10 mM MgATP[d] | $>1 \times 10^3$ |

[a] ATPase assays were conducted as in legend to Figure 4. Where preincubation of the Pgp with inhibitor was required (described in footnotes), ATP was added to start the reaction. [b] Purified Pgp (6.1 $\mu$g/mL) was preincubated for 10 min at 37 °C with inhibitor in 40 mM Tris-Cl, 0.1 mM EGTA, 0.25 M sucrose, 50 $\mu$M verapamil. The pH was 7.4 with vanadate and DCCD, 9.0 with FITC. With FITC, the Pgp was made free of DTT as in legend to Figure 6. [c] Purified Pgp was preincubated with NEM as in Figure 6 legend. [d] Purified Pgp was preincubated with NBD-Cl as in Figure 7 legend.

The second goal of this work was to characterize the purified Pgp with specific reference to the catalytic activity of the nucleotide-binding sites. The purified Pgp displayed substantial and stable verapamil-stimulated ATPase activity and it was found that the kinetic parameters for this activity ($K_M$, $k_{cat}$, $k_{cat}/K_M$) were similar to those reported previously for Pgp in CR1R12 cell plasma membranes by Al-Shawi and Senior (1993) and Al-Shawi *et al.* (1994). Accordance between the catalytic characteristics of the purified Pgp and those of membrane-bound Pgp was further confirmed by the similarity of pH dependence and magnesium versus calcium specificity and the parallel susceptibility to different inhibitors. For example, the concentrations of vanadate required for 50% inhibition in membrane-bound and purified Pgp were very similar, and NBD-Cl and NEM inactivated the ATPase activity of purified Pgp with similar potency as toward Pgp in plasma membranes, with ATP giving strong protection against inactivation by either reagent (Figures 6 and 7). These results together demonstrate that the catalytic sites retained their native folded conformation and catalytic characteristics in the purified Pgp, and therefore the purified Pgp preparation is fully satisfactory for future structural and functional studies of the catalytic nucleotide-binding site(s). The substrate nucleotide specificity of purified Pgp was quite broad (Table 1) and a range of ATP analogs, including potential photoaffinity and fluorescent probes of the catalytic sites, were found to be good substrates. ADP, AMPPNP, and ATP$\gamma$S were competitive inhibitors, with relatively low potency. Therefore the conclusions drawn by Al-Shawi *et al.* (1994) for the plasma-membrane-bound Pgp, i.e. that the catalytic sites are of low affinity, tolerant of modification of the adenine ring, and with no indication of a high affinity ATP-binding site involved in catalysis, appear to apply equally well to purified Pgp. It is of interest to note that many of the catalytic features seen here with purified Pgp resembled those of the MalK protein as described recently by Morbach *et al.* (1993), e.g. pH dependence, Mg versus Ca specificity, broad substrate specificity, weak competitive inhibition by ADP and ATP$\gamma$S, and profile of inhibition by various reagents.

The ATPase activiy of purified Pgp was stimulated by a range of compounds known from the extensive previous literature to interact at the transport substrate sites of Pgp in whole cells (Table 2). However, colchicine, vinblastine, vincristine, adriamycin, and daunomycin did not stimulate, although each of these drugs stimulates ATPase activity of Pgp in CR1R12 plasma membranes (Al-Shawi & Senior,

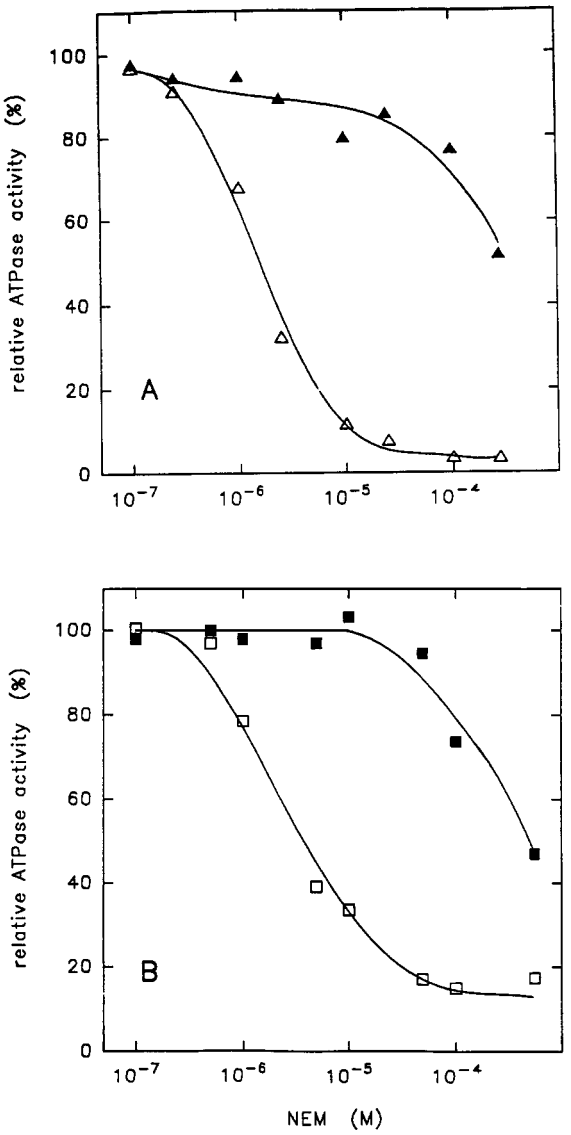


FIGURE 6: Inhibition of plasma membrane or purified P-glycoprotein ATPase activity by *N*-ethylmaleimide. (A) Plasma membranes were preequilibrated in buffer containing 40 mM Tris-Cl, pH 7.4, 0.25 M sucrose, 0.1 mM EDTA by passage through 1-mL centrifuge columns containing Sephadex G-50 (Penefsky, 1977) and adjusted to a final protein concentration of 55 μg of membrane protein/mL. (B) Proteoliposomes containing purified P-glycoprotein were first freed of DTT by dialysis against degassed, $N_2$-saturated 50 mM Tris-Cl pH 7.4. Removal of DTT was checked with Ellman's reagent. The proteoliposomes were then dialyzed against 40 mM Tris-Cl, pH 7.4, 0.1 mM EDTA, and adjusted to a final protein concentration of 55 μg/mL. NEM was added at various concentrations and samples were incubated under an $N_2$ atmosphere for 10 min at 37 °C in the presence (▲, ■) or absence (△, □) of 10 mM MgATP, then diluted 1:20 into ATPase medium, and assayed as described in Methods; 100% activity was defined as that found in absence of added NEM.

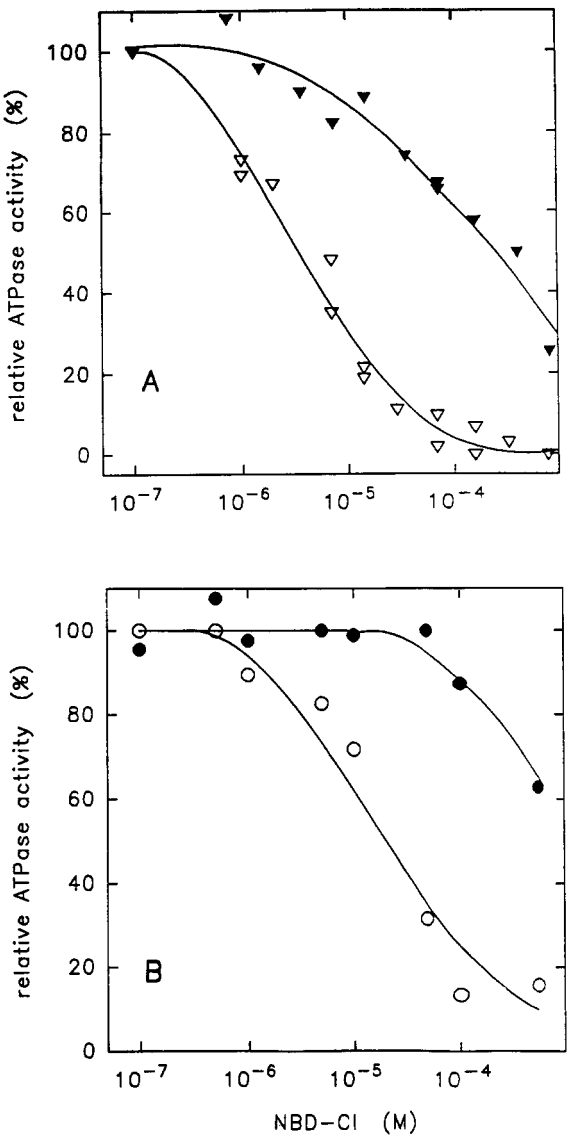


FIGURE 7: Inhibition of plasma membrane or purified P-glycoprotein ATPase activity by NBD-Cl. (A) Plasma-membrane-bound P-glycoprotein; (B) purified P-glycoprotein in proteoliposomes. Conditions were as in Figure 6. (▼, ●), plus 10 mM MgATP; (▽, ○), absence of MgATP.

1993). It was noted that high concentrations of these drugs did inhibit ATPase activity of purified Pgp, as was reported earlier with plasma-membrane-bound Pgp, suggesting that there was interaction between the drugs and purified Pgp, at least at high concentrations.

Several workers (Sharom *et al.*, 1993; Doige *et al.*, 1993; Zordan-Nudo *et al.*, 1993; Al-Shawi & Senior, 1993) have reported and discussed the effects of detergent molecules and of the lipid environment on interaction between activators and the the drug-binding sites of Chinese hamster Pgp, located in the hydrophobic membrane-embedded domain of the molecule (reviewed in Gottesman & Pastan, 1993). The lipid environment is also likely to be of considerable importance for correct signal transmission between the transport substrate sites and the catalytic sites, which is presumably responsible for the activation of ATPase activity.

The purified Pgp preparation described here was reconstituted in *E. coli* lipid, which might not optimally mimic the natural environment of the membrane-embedded part of the protein. After this work was submitted for publication, a report appeared (Shapiro & Ling, 1994) in which Chinese hamster Pgp was obtained at about 90% purity, using a different purification procedure from that described here. In general the data reported in that paper and those described here are in agreement. However, the preparation of Shapiro and Ling, which was reconstituted in proteoliposomes using a crude brain lipid preparation, did show significant stimulation of ATPase activity by vinblastine and modest stimulation by colchicine and daumomycin. We have subsequently shown that the Pgp purified as described here, but reconstituted using either crude brain lipid or crude liver lipid, showed significant stimulation of ATPase activity by vinblastine, daunomycin, and colchicine (Urbatsch & Senior, manuscript in preparation). It is clear therefore that the lipid environment in which Pgp is embedded can markedly affect the degree of drug

stimulation of ATPase activity observed. It bears reiteration, however, that there is apparently no straightforward correlation between stimulation of ATPase activity and transport, since it was shown that several drugs that did not stimulate ATPase activity in a partially-purified, reconstituted preparation of Pgp nevertheless did appear to be transported or to compete effectively for transport (Sharom *et al.*, 1993). The mechanism of coupling between the catalytic sites and the drug-binding sites, and its regulation, will clearly be an important field of future studies.

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