Characterization of the Adenosine Triphosphatase Activity of Chinese Hamster P-glycoprotein*

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A multidrug-resistant Chinese hamster ovary cell line (CR1R12) was obtained which constitutively expresses P-glycoprotein, up to 32% by weight of plasma membrane protein. CR1R12 plasma membranes had high, drug-activated ATPase activity referable to P-glycoprotein. The specific ATPase activity in the presence of verapamil was calculated to be ~9 μmol/min/mg (~21 s⁻¹) at 37 °C, pH 7.4. Kᵢ ATb was 1.4 mm, and ADP and 5'-adenylyl imidodiphosphosphate were competitive inhibitors with Kᵢ values 0.35 and 0.44 mm, respectively. 2'-dATP was a good substrate, GTP and ITP were real but poor substrates, and ADP and AMP were not hydrolyzed. Optimal pH for ATP hydrolysis was 7.3. MgATP was the preferred substrate, and Ca₂⁺ATP was hydrolyzed very weakly. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) covalently labeled the P-glycoprotein, and incorporation of 1.1 mol of NBD-Cl/mol of P-glycoprotein gave 100% inactivation. ATP protected against NBD-Cl inactivation. N-Ethylmaleimide was a potent inhibitor in the absence of ATP, and in its presence significant protection from inhibition could be achieved. Vanadate and fluoroaluminate were also strong inhibitors. The plasma membranes from CR1R12 cells should provide material for purification and reconstitution of P-glycoprotein and for screening of potential "multidrug-reversal" reagents by enzymic assay.

Multidrug resistance is a condition encountered in cancer patients in which the tumor becomes resistant to a variety of cytotoxic chemotherapeutic agents (Riordan and Ling, 1985). Usually it involves amplification of "multidrug resistance" genes leading to overexpression of P-glycoprotein in the plasma membranes. P-glycoproteins are approximately 1280 amino acids in length and have apparent molecular masses of 130–170 kDa, depending on the state of glycosylation (reviewed in Endicott and Ling, 1989; Gottesman and Pastan, 1988; Ling, 1992). The amino acid sequences of P-glycoproteins exhibit strong homologies to nucleotide-binding proteins and to a large superfamily of membrane-associated transport proteins (Juranka et al., 1989). P-glycoprotein is a tandemly duplicated molecule, each half of which appears to have six transmembrane nonpolar α-helices and one cytoplasmically sided nucleotide-binding domain. This proposed topology seems to be confirmed by various biochemical approaches (Greenberger et al., 1987; Yoshimura et al., 1989).

It has been demonstrated that in multidrug-resistant cell lines drug efflux is an energy-dependent process that requires ATP (reviewed in Bradley et al., 1988). P-glycoprotein-containing plasma membranes vesicles exhibit ATP-dependent drug transport (Horio et al., 1988) and bind the ATP analog 8-azido-ATP (Cornwell et al., 1987; Georges et al., 1990). Site-directed mutagenesis of either or both of the putative ATP-binding domains inhibited the drug transport function of P-glycoprotein (Azzaria et al., 1989). All of these results strongly suggest that P-glycoprotein is an ATP-dependent drug-exporting transport protein.

Several attempts have been made to measure ATPase activity of P-glycoprotein and to study its modulation by transport substrates. Hamada and Tsuruo (1988a, 1988b) succeeded in demonstrating ATPase activity associated with immunoperoxidase-purified human P-glycoprotein and in immunoprecipitated protein extracts, but the activities they reported were unrealistically low (turnover = 0.003 and 0.12 s⁻¹, respectively) and could not account for the drug transport rates and ATP consumption seen in multidrug-resistant cells. Thus, substantial inhibition and/or denaturation probably occurred during purification and exposure to detergents. Similarly, immunoprecipitated human P-glycoprotein/β-galactosidase fusion protein showed a relatively low specific activity (turnover = 0.77 s⁻¹) (Shimabuku et al., 1992). Recently, Sarkadi et al. (1992) expressed human P-glycoprotein in Sf9 insect cells and found a substantially higher, drug-stimulated ATPase activity in the plasma membranes. P-glycoprotein was calculated to constitute about 3% by weight of the total plasma membrane proteins, and by extrapolation it was calculated that the specific ATPase activity of P-glycoprotein was 3–5 μmol of ATP hydrolyzed/min/mg (= turnover of 7–12 s⁻¹). Here we report the preparation of plasma membranes containing up to 32% by weight P-glycoprotein from multidrug-resistant Chinese hamster ovary cells. These membranes have substantial ATPase activity, which is shown to be referable to P-glycoprotein, and is further characterized.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—The following Chinese hamster ovary cell lines were used: the drug-sensitive parental auxotroph AUXB1 (McBurney and Whitmore, 1974), the multidrug-resistant line CHCR5 (Bech-Hansen et al., 1976), and the highly multidrug-resistant line CR1R12 developed here. Cell lines were routinely passaged and grown on solid support in α-MEM medium with glutamine, ribonucleosides, and deoxyribonucleosides (GIBCO catalog No. 410-1900EB), 50 units/ml penicillin G plus 50 μg/ml

1 The abbreviations used are: α-MEM, α-minimal essential medium; AMP-PNP, 5'-adenylyl imidodiphosphate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; CHAPSO, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; DCCD, N,N'-dicyclohexylcarbodiimide; DTT, dithiothreitol; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; Me₃SO, dimethyl sulfoxide.

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streptomycin sulfate (complete α-MEM), supplemented with 10% fetal calf serum. In the case of CR1R12 cells, the medium was supplemented with 5 μg/ml colchicine to prevent reversion.

Selection for Increased Expression of P-glycoprotein—CH²C5 cells were grown on multiple 15-cm dishes in the presence of 8 μM of colchicine. The cell growth and protein concentration was increased incrementally by 5 μg/ml steps, achieving stable growth at each new concentration. At a final concentration of 30 μg/ml, individual colonies were chosen and subjected to two purification cycles.

Cytotoxicity Assays—Colony formation in the presence of different drugs was assayed by the method of Gottman (1987). Briefly, 400 or 40,000 cells were plated into wells of 12-well tissue culture dishes. Each well contained 2 ml of complete α-MEM plus 10% fetal calf serum with 3 mM MgCl₂ and DNase I. Cells were lysed by doubling the medium volume every 24 h. At the end of growth at 37°C the colonies were stained with 0.5% methylene blue and counted manually on an inverted microscope.

Immunoblot Analysis of P-glycoprotein Content of Cells—For analysis by a dot blot procedure, cells were suspended at 1 mg of protein/ml in buffer A (0.25 M sucrose, 20 mM Tris·Cl, pH 7.4, 1 g/liter DTT, 2 g/liter para-aminobenzoamide, 2 mM phenylmethylsulfonyl fluoride, 10 g/liter 6-aminopurine acid, and 1 mM EGTA), supplemented with 3 mM MgCl₂ and DNAse I. Cells were lysed on ice with a SoniCell Disruptor (Branson Co.) (90 s, 0°C, setting 4), and incubated at 40°C for 10 min. Then, the cell fraction was used instead of phosphate-buffered saline and 30 mM Tris·Cl, pH 7.4, was present in the sucrose gradients. The plasma membrane fraction accumulated at the 16/31% (w/v) interface. Percent membrane protein present. This was done by plotting, for each lane, the protein content was calculated from the ratio of slopes of the two lines.

Determination of Nuclear Cell Disruption—Nuclear cell disruption was performed with the "TMB Membrane Peroxidase Substrate Kit" (Kirkegaard and Perry Labs Inc.). Similarly, "Western" blots of protein gels were developed as above. Immunoblots were scanned by laser densitometry (LRB Ultroscan XL, interfaced with a computer).

Plasma Membrane Preparation—Cell suspensions were grown in 1-liter spinner flasks at 37°C in a 5% CO₂ incubator. Complete α-MEM was supplemented with 10% "Fetal Clone" (HyClone), and 3% dialyzed fetal bovine serum every 24 h. At the end of log phase, cells were harvested and washed with 25 mM HEPES, pH 7.4, 10 mM NaHCO₃, 1 mM K₂HPO₄, 1 mM MgSO₄, 1 mM CaCl₂, 125 mM NaCl, 11 mM glucose, spun down, and resuspended at 1.5 x 10⁶ cells/ml in ice cold buffer A (above). All subsequent procedures were performed at 4°C. The cells were equilibrated for 30 min with 450 p.s.i. N₂ gas in a Parr cell-disruption apparatus. On release of pressure, nearly all cells were disrupted. Preparation of the plasma membrane fractions from discontinuous sucrose gradients was performed according to Roriodan and Ling (1979) except that buffer A was used instead of phosphate-buffered saline and 30 mM Tris·Cl, pH 7.4. Preparations between 1 and 3% sucrose were used. The plasma membrane fraction accumulated at the 16/31% (w/v) interface. Plasma membranes were resuspended in buffer A and stored at ~70°C. The ATPase activity was stable for at least 1 year.

Quantification of P-glycoprotein in Plasma Membranes—Increasing amounts of plasma membrane protein (40-40 μg) were run in adjacent lanes on 10% SDS-PAGE and were stained with Coomassie Blue R-250. Destained gels were scanned by laser densitometry, and P-glycoprotein content was estimated as a fraction of the total membrane protein present. This was done by plotting, for each lane, the integrated stain intensity of the P-glycoprotein peak alone, and of all the peaks summed together, against the amount of protein loaded as determined by the bicinchoninic acid assay. The percent P-glycoprotein content was calculated from the ratio of slopes of the two lines. The analysis was done over a range in which the optical density was a linear function of the protein present, and lanes containing bovine serum albumin were also run to ensure the linearity of staining. Computer integration of stain intensities and manual determination by paper weighing gave essentially the same results.

Production of Polyclonal Anti-P-glycoprotein Antibodies—CR1R12 plasma membranes were run on preparative 10% SDS-polyacrylamide gels and were stained and destained. The P-glycoprotein band was excised, washed extensively with sterile 0.9% saline solution, and the gel was crushed and passed through a 27-gauge needle with an equal volume of saline. Seventy μg of P-glycoprotein was emulsified with an equal volume of Freund's complete adjuvant and injected intradermally on the back of a New Zealand white rabbit. After 6 weeks, and monthly thereafter, 30-70 μg of P-glycoprotein was injected subcutaneously without added adjuvant. Antibody production was monitored by enzyme-linked immunosorbant assay and Western blots done according to Perlin and Senior (1985) using goat anti-rabbit IgG-peroxidase as the secondary antibody. Anti-P-glycoprotein specificity of the sera was established by Western blots using plasma membranes from AUXB1, CH²C5, and CR1R12 cells.

Assay of ATPase Activity—ATPase activity was measured as described in the tables or figure legends. Ten μg or less of membrane protein was incubated in 200 μl of ATPase assay medium, which unless otherwise specified included 2 mM ouabain (to eliminate Na,K-ATPase activity) and 100 μM EGTA (to eliminate Ca-ATPase). Reactions were started by the addition of MgATP or membranes and were stopped by injecting 50-75 μl of reaction mix into 1 ml of 20 mM HSO₄ on ice. Pi released was determined by the method of Van Veldhoven and Mannaearts (1987). Activities were calculated from the initial linear rate of Pi production. Activators or inhibitors were dissolved in dimethyl sulfoxide (Me₂SO) and the final concentration of Me₂SO was <2% (v/v).

Quantitation of Radioactivity of Protein Bands in Polyacrylamide Gels—Gel slices containing protein bands were incubated in 200 μl of 30% H₂O₂ for 3 h at 70°C then counted in 10 ml of Amerash ACS scintillant with 50 μl of 10% ascorbic acid. For fluorography, gels were destained and incubated for 1 h in 125 ml of Amplify dried on paper support, and fluorographs taken on preflashed x-ray film at ~70°C. Immunoblots were fluorographed by incubating the nitrocellulose sheets with Amplify for 30 min. The sheets were then air-dried and developed as above.

Routine Methods—SDS-PAGE was according to Laemmli (1970). Samples containing protein bands were not boiled prior to electrophoresis because boiling caused poor resolution and aggregation of P-glycoprotein (Greenberger et al., 1988). Instead samples were dissolved in 7%/w/v SDS, 25%/v/v glycerol, 0.125 M Tris·Cl, pH 6.8, 40 mM DTT and incubated at room temperature for 30 min prior to loading. Transfer of proteins from SDS-polyacrylamide gels to nitrocellulose sheets for Western blots was performed as in Perlin and Senior (1985). Protein assays were done in the presence of 1% SDS by the BCA (bicinchoninic acid) method (Smith et al., 1985) using bovine serum albumin as a standard with appropriate buffer blanks. Concentrations of ionic species of magnesium, calcium, and ATP were measured according to Fabiato and Fabiato (1979). Cytochrome oxidase activities were measured by the method of Gibson and Hill (1983).

Other Materials—[U-¹⁴C]NBD-Cl was from Research Products International. Tissue culture material was purchased from GIBCO and general chemicals from Sigma.

RESULTS

Selection of a Highly Multidrug-resistant, P-glycoprotein-overproducing Cell Line

The CH²C5 cell line was originally derived from AUXB1 cells as a multidrug-resistant derivative (Bech-Hansen et al., 1976). Here, we took CH²C5 cells and subjected them to incremental increases in colchicine concentration. Nineteen derivative sublines were established and assessed for multidrug-resistance, content of P-glycoprotein, constitutive overexpression of P-glycoprotein, and growth characteristics in suspension culture and on solid support. One line with desired characteristics was chosen and named CR1R12. Table I shows the enhanced multidrug resistance of colony formation of CR1R12 cells as compared to AUXB1 and CH²C5 cells. We

<table>
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<th>TABLE I</th>
<th>Drug sensitivity of AUXB1, CH²C5, and CR1R12 cell lines</th>
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<tbody>
<tr>
<td>Cell</td>
<td>Colchicine Daunomycin Vinblastine</td>
</tr>
<tr>
<td>AUXB1</td>
<td>0.013 (1) 0.021 (1) 0.001 (1)</td>
</tr>
<tr>
<td>CH²C5</td>
<td>4.8 (570)  2.3 (110)  0.14 (190)</td>
</tr>
<tr>
<td>CR1R12</td>
<td>20 (1540) 14 (670) 1.2 (1200)</td>
</tr>
</tbody>
</table>

*ED₅₀ is the concentration of drug in μg/ml required to reduce colony formation to 50% of that seen in the absence of drug. See "Experimental Procedures" for details.

Values in parentheses show resistance relative to AUXB1 cells.
also carried out growth tests in suspension culture in the presence of doxorubicin and showed CR1R12 cells are 300-fold more resistant to this drug than AUXB1 cells. Table II demonstrates that CR1R12 cells exhibit rapid growth in suspension culture (column 1), grow to an acceptable final cell density (column 2) and show a high content of constitutively expressed P-glycoprotein in whole cell lysates (column 3).

Plasma membranes were prepared from the drug-sensitive parental line AUXB1 and from the multidrug-resistant lines CHPC5 and CR1R12. Fig. 1 shows a Coomassie Blue-stained gel and anti-P-glycoprotein immunoblot (using the monoclonal antibody C219) of the three types of plasma membranes. It is clear that CR1R12 has the most P-glycoprotein.

The P-glycoprotein usually ran as a single band with an estimated molecular mass of 152 kDa. This is further demonstrated in Fig. 2, lane A, which shows a single P-glycoprotein band on a Western blot developed with polyclonal rabbit antibody anti-P-glycoprotein serum. Fig. 2 also shows a densitometric scan of CR1R12 plasma membranes run on SDS-PAGE, from which it can be seen that P-glycoprotein constitutes a considerable fraction of the total plasma membrane protein (28% in this preparation). Table II (column 4) summarizes the P-glycoprotein content of plasma membrane preparations. CR1R12 plasma membranes appear to have the highest P-glycoprotein content of any cell line reported to date. It should be noted that whereas CR1R12 whole cell lysates had 5-fold more P-glycoprotein content of CHPC5 on a per milligram of total protein basis, the CR1R12-purified plasma membranes had only about 2-fold. This may be due to morphological differences; the CR1R12 cells were longer than CHPC5 cells when grown on solid support.

**ATPase Activity Associated with P-glycoprotein**

ATPase activities associated with the plasma membranes of the three cell lines are shown in Fig. 3 and are given in Table III. Unless otherwise indicated ATPase activities were assayed in the presence of 2 mM ouabain and 100 μM EGTA to eliminate any Na,K-ATPase and Ca-ATPase activities. Ouabain and EGTA are known not to interfere with P-glycoprotein-dependent drug transport (Kamimoto et al., 1989; Horio et al., 1988). Both CHPC5 and CR1R12 plasma membrane preparations have substantial ATPase activities, approximately in proportion to their P-glycoprotein content whereas the P-glycoprotein-deficient plasma membranes of the parental line AUXB1 had very low ATPase activity (Fig. 3 and Table III, column 1). Different plasma membrane preparations from CR1R12 cells contained variable amounts of P-glycoprotein (13-32% of total membrane protein, Fig. 3), and there was a good correlation between the ATPase activity seen and measured P-glycoprotein content of the different preparations. The average specific activity of CR1R12 plasma membranes of Fig. 3 was 3.4 ± 0.2 μmol/min/mg of P-glycoprotein (S.E., n = 12). Colchicine (100 μM) stimulated the ATPase activity of CHPC5 and CR1R12, but had little effect on AUXB1 plasma membranes (Table III, column 2). The stimulation was ~1.5-fold for CR1R12 membranes. Small amounts of Triton X-100 (0.1–0.2 mg/mg of membrane protein) were included to permeabilize the membranes to ATP and reveal any latent ATPase activity. It was found that Triton X-100 did stimulate the ATPase activities by 2-fold (Table III, column 3) suggesting that the plasma membrane preparations were approximately 50% inside out. Above 0.25 mg/mg of protein, Triton X-100 began to inhibit ATPase. Inclusion of colchicine with Triton X-100 led to a further slight activation (Table III, column 4). As will be shown later, Triton X-100 was found to interfere with the activation of ATPase activity by drugs.

In data not shown here we also found that the detergents CHAPS and CHAPSO, in the presence of 20% (v/v) glycerol as protectant osmolyte (Maloney and Ambukar, 1989), also stimulated CR1R12 plasma membrane ATPase activity by up to 2-fold, at an optimal detergent concentration of 15 mM. Seventy % of the membrane protein was solubilized under these conditions. Hypotonic osmotic disruption of membranes by removal of the sucrose from the ATPase assay medium increased ATPase activity by up to 1.9-fold. Furthermore, 5% Me2SO added as a membrane-disrupting agent also increased the ATPase activity by 2-fold. These results support the suggestion that the membranes were sealed as isolated and had a ~50% latency due to half of the catalytic sites being inaccessible to ATP. These observations indicate that the membranes may be useful in future drug transport studies (see e.g., Lelong et al., 1992).

Na,K-ATPase activity was similar in plasma membrane preparations from all three cell types (Table III, column 5) and was abolished by 2 mM ouabain and exclusion of Na+ and K+. There was very low Ca-ATPase activity (see below) in any of the membranes. Ecto-ATPase activity in the CR1R12 membranes had a maximal level of 0.018 μmol/min/mg when assayed as described by Lin and Guidotti (1989). Plasma membranes from CR1R12 cells had 1.4% of the specific cytochrome oxidase activity of the mitochondrial fraction, and thus are expected to be essentially free of mitochondrial ATPase.

Summarizing this section, we find a large ATPase activity in plasma membranes of multidrug-resistant cell lines that is not due to Ca-ATPase, Na,K-ATPase, ecto-ATPase, or mitochondrial ATPase and is correlated with the amounts of P-glycoprotein present in the membranes. The specific ATPase activity is similar to that reported for human P-glycoprotein expressed in SF9 cells (Sarkadi et al., 1992), but the total ATPase activity in CR1R12 plasma membranes is an order of magnitude higher due to the 10-fold higher relative content of P-glycoprotein.

### TABLE II

<table>
<thead>
<tr>
<th>Characteristics of AUXB1, CHPC5, and CR1R12 cell lines</th>
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<tbody>
<tr>
<td><strong>Cell line</strong></td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>AUXB1</td>
</tr>
<tr>
<td>CHPC5</td>
</tr>
<tr>
<td>CR1R12</td>
</tr>
</tbody>
</table>

a Determined by immunoblot analysis (see “Experimental Procedures”).

b Determined by laser densitometry of Coomassie Blue-stained plasma membranes run on SDS-PAGE (see “Experimental Procedures”).

c No P-glycoprotein was detected.

d Maximal values seen are reported. The average P-glycoprotein content of CR1R12 plasma membranes was 21 ± 6% (S.D., n = 12).
Further Characterization of P-glycoprotein ATPase Activity

Drug Activation—Fig. 4A shows the effect of various drugs on CR1R12 plasma membranes ATPase activity. These drugs are thought to be transported and have been shown to compete for the azidopine binding site of P-glycoprotein (Horio et al., 1988; Germann et al., 1990). The calcium channel blocker verapamil enhanced ATPase activity maximally by 4.9-fold, with half-maximal enhancement at 0.9 µM (Fig. 4A). Other drugs were less effective, thus vinblastine, colchicine, and daunomycin activated maximally by 1.9-, 1.8-, and 1.6-fold, respectively. All these drugs were effective within a concentration range in which they interacted with the P-glycoprotein transport function (Horio et al., 1988; Busche et al., 1989). Our results are similar to those obtained by Sarkadi et al. (1992) for human P-glycoprotein ATPase. In the presence of Triton X-100 (Fig. 4B), the activation by drugs was diminished, and ATPase activity became inhibited at higher concentrations of the drugs (note the vertical axes scale change in Fig. 4, B versus A). This may be due to an interference of detergent with the drug-binding site (Hamada and Tsuruo, 1988b; Sarkadi et al., 1992). When CR1R12 plasma membranes were solubilized in the presence of 15 mM CHAPS and 20% (v/v) glycerol (see above), 5 µM verapamil stimulated the ATPase activity of the detergent-treated membranes by only 1.2-fold, showing this may be a widespread effect of detergents.

Kinetic Parameters—Kinetic parameters of the ATPase activity associated with the Chinese hamster P-glycoprotein are reported in Table IV. KM for MgATP hydrolysis is rather high (1.4 mM). This is higher than the values reported for human P-glycoprotein (Hamada and Tsuruo, 1988b; Sarkadi et al., 1992) and is significantly higher than KM values for mitochondrial ATPase, NaN-K-ATPase, sarcoplasmic reticulum or erythrocyte Ca-ATPase, and gastric H,K-ATPase (Hammes and Hilborn, 1971; Kanazawa et al., 1970; Vianna, 1975; Cabantchik et al., 1975; Ray and Forte, 1976). Addition of 120 µM colchicine increased Vmax by 2.4-fold and shifted the KM for MgATP to 3 mM. Ten µM verapamil increased Vmax by 4.7-fold to 9 µmol P/mg P-glycoprotein/min but the KM was unchanged. Me2SO was used as the solvent for verapamil and has been shown above to expose latent P-glycoprotein activity in intact membranes. Thus, the kinetic parameters of verapamil-treated membranes were the same as osmotically disrupted and verapamil treated membranes (Table IV).

pH Dependence—Fig. 5 illustrates the pH dependence of ATPase activity. In the absence of verapamil maximal activity occurred over a broad range. This result agrees with the result obtained for the immunoprecipitated human P-glycoprotein (Hamada and Tsuruo, 1988b). When verapamil was present the pH optimum was at 7.3. The enzyme was essentially inactive at pH 4.5 and 10.

Nucleotide Specificity—Table V shows the hydrolysis of various magnesium nucleotides by CR1R12 plasma membranes. There was a clear selectivity for ATP over ITP and GTP. Under all conditions used, ITP was a better substrate than GTP. Recently, it has been found that GTP was a good
The presence of 10 μM verapamil (Table IV). In this last respect P-glycoprotein was similar to the Na,K-ATPase, but with a specific protein present as P-glycoprotein in this table was AUXBl, 0%; CHRC5, 12%; CRlR12, 28%.

7.4. Plasma membrane concentrations were 10–50 pg/ml. Activity is given as pmol Pi released/mg membrane protein/min. The ATPase was hydrolyzed very poorly by Na,K-ATPase (Hegyvary and Post, 1971). Energy source for drug transport by human P-glycoprotein, whereas ITP was less effective (Lelong et al., 1992), and it differs in the hydrolysis of ITP and GTP which were sensitive to ATP depletion. AMP was also not hydrolyzed up to 10 mM, and had no effect on the ATPase activity at 5 mM AMP. Mg2+-dATP was an excellent substrate with a Vmax of 6.7 μmol/mg of P-glycoprotein/min and a Km of 1.1 mM in the presence of 10 μM verapamil (Table IV). In this last respect P-glycoprotein was similar to the Na,K-ATPase, but it differs in the hydrolysis of ITP and GTP which were hydrolyzed very poorly by Na,K-ATPase (Hegyvary and Post, 1971).

Magnesium Versus Calcium Specificity—The metal ion specificity of P-glycoprotein ATPase was investigated in Fig. 6. It can be seen that magnesium is a much better activator of the ATPase than calcium (Fig. 6A) and that up to 50 mM free Mg2+ did not significantly inhibit the enzyme. This is clearly different from the effect of free Mg2+ on Ff-ATPase (Al-Shawi et al., 1988), sarcoplasmic reticulum Ca-ATPase (Bishop and Al-Shawi, 1987), and Na,K-ATPase (Robinson, 1974). Fig. 6B shows enzyme activity as a function of cation-ATP complex concentrations. Maximal Ca-ATPase activity was at 5.5 mM Ca-ATP and half-maximal Ca-ATPase activity was at 2.2 mM, but a free Ca2+ of greater than 0.17 mM inhibited the enzyme. When 0.1 mM EGTA was added in the absence of divalent cations, with 10 mM NaATP as substrate, there was no measurable activity, indicating that divalent cations are required. The cation specificities observed here were qualitatively similar to those seen for the purified human P-glycoprotein (Hamada and Tsuruo, 1988b).

### Table III

**ATPase activities of plasma membrane preparations**

ATPase activity was assayed at 37 °C in 0.25 M sucrose, 5 mM ATP, 2.5 mM MgCl2, 0.1 mM EGTA, 2 mM ouabain, and 40 mM Tris-Cl, pH 7.4. Plasma membrane concentrations were 10–50 pg/ml. Activity is given as μmol P released/mg membrane protein/min. The % of membrane protein present as P-glycoprotein in the CR1R12 plasma membranes was determined at 37 °C in 0.25 M sucrose, 15 mM KCl, 60 mM NaCl, 5 mM ATP, 2.5 mM MgCl2, 0.1 mM EGTA, 40 mM Tris-Cl, pH 7.4, and 5 μg of Triton X-100/ml with and without 2 mM ouabain. The Triton X-100 was included to permeabilize the membranes to ATP and at the concentration used is without effect on the Na,K-ATPase. The activities given represent the difference in the presence and absence of ouabain.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Membrane ATPase</th>
<th>Membrane ATPase + colchicine*</th>
<th>Membrane ATPase + Triton X-100+ colchicine</th>
<th>Membrane ATPase + Triton X-100+ colchicine</th>
<th>Na,K-ATPase+ colchicine</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUXBl</td>
<td>0.013</td>
<td>0.016</td>
<td>0.017</td>
<td>0.017</td>
<td>0.022</td>
</tr>
<tr>
<td>CHRC5</td>
<td>0.26</td>
<td>0.47</td>
<td>0.47</td>
<td>0.47</td>
<td>0.51</td>
</tr>
<tr>
<td>CRlR12</td>
<td>0.48</td>
<td>0.71</td>
<td>0.93</td>
<td>0.93</td>
<td>1.01</td>
</tr>
</tbody>
</table>

*ATPase was assayed in the presence of 120 μM colchicine.

†ATPase was assayed in the presence of 5 μg of Triton X-100/ml.

‡Na,K-ATPase activity was determined at 37 °C in 0.25 M sucrose, 15 mM KCl, 60 mM NaCl, 5 mM ATP, 2.5 mM MgCl2, 0.1 mM EGTA, 40 mM Tris-Cl, pH 7.4, and 5 μg of Triton X-100/ml with and without 2 mM ouabain. The Triton X-100 was included to permeabilize the membranes to ATP and at the concentration used is without effect on the Na,K-ATPase. The activities given represent the difference in the presence and absence of ouabain.

![Diagram](image_url)

Fig. 4. Effects of drugs on ATPase activity in CR1R12 plasma membranes. A, ATPase activities were measured as described in Table III. The membrane concentration was 50 μg of membrane protein/ml of ATPase assay medium. 100% activity here was 1.5 μmol of ATP hydrolyzed/mg of P-glycoprotein/min. O, verapamil; □, colchicine; Δ, vinblastine; ●, daunomycin. B, ATPase activities were measured in the presence of 5 μg of Triton X-100/ml. 100% activity here was 3.1 μmol of ATP hydrolyzed per mg of P-glycoprotein/min. Symbols are as in A.

Energy source for drug transport by human P-glycoprotein, whereas ITP was less effective (Lelong et al., 1992), and it will be interesting to investigate the apparent differences in specificity observed above. ADP was not hydrolyzed (Table V) and was found to behave as a classical competitive inhibitor with a KI of 0.35 ± 0.15 mM (±S.D.). This fact, together with the high Km for ATP, may explain why drug transport is very sensitive to ATP depletion. AMP was also not hydrolyzed up to 10 mM, and had no effect on the ATPase activity at 5 mM AMP. Mg2+-dATP was an excellent substrate with a Vmax of 6.7 μmol/mg of P-glycoprotein/min and a Km of 1.1 mM in the presence of 10 μM verapamil (Table IV). In this last respect P-glycoprotein was similar to the Na,K-ATPase, but it differs in the hydrolysis of ITP and GTP which were hydrolyzed very poorly by Na,K-ATPase (Hegyvary and Post, 1971).

### Reaction of P-glycoprotein with NBD-Cl and Inhibition of ATPase Activity

NBD-Cl has been used extensively as a covalent inhibitor of transport ATPases (see e.g. Ferguson et al., 1975a, 1975b; Cantley et al., 1978a; Puopolo and Forgac, 1990). Fig. 7A illustrates the inhibitory effect of NBD-Cl on CR1R12 plasma membrane ATPase activity. The inhibition was relatively fast for the conditions used, and it was found to be a function of the concentrations of NBD-Cl and membranes (not shown). NBD-Cl inhibited more effectively at higher pH, and there was no inhibition at pH 5.5 (Fig. 7B). ATP offered some protection against NBD-Cl inhibition of ATPase activity. For a 10-min reaction under conditions as in Fig. 7A, 50% inhibition was achieved with 6.4 μM NBD-Cl. Inclusion of 10 mM MgATP increased the 50% inhibitory concentration to 160 μM NBD-Cl. Fig. 8A illustrates the kinetics of reaction of [14C]NBD-Cl with CR1R12 plasma membranes at pH 7.4, and Fig. 8C shows the pH dependence of the reaction. P-glycoprotein was the major protein band that was labeled, and this labeling increased as the pH was raised. Fig. 8B shows AUXB1 plasma membranes reacted under similar conditions. A comparison of Fig. 8B with A and C indicates that all of the same minor bands were labeled in AUXB1 and CR1R12 membranes, with the major difference being the extensive labeling of P-glycoprotein in the CR1R12 membranes. A correlation between amount of [14C]NBD-Cl incorporated into the P-glycoprotein band in CR1R12 membranes and inhibition of ATPase activity as a function of either time (Fig. 9) or of pH (not shown) was seen, whereas the [14C]NBD-Cl incorporated into the minor bands did not correlate with ATPase inhibition.
P-glycoprotein ATPase Activity

**TABLE IV**

**MgATPase activity in CR1R12 plasma membranes**

ATPase activities of CR1R12 plasma membranes were assayed at 37 °C in 0.25 M sucrose, 40 mM Tris-SO₄, pH 7.4, 0.1 mM EGTA, and 2 mM ouabain. The concentration of MgATP was varied from 50 μM to 20 mM and the data were fit to the Michaelis-Menten equation by nonlinear least squares regression analysis.

<table>
<thead>
<tr>
<th>Additions to assay</th>
<th>Vₘₐₓ</th>
<th>Kₘ MgATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Colchicine (120 μM)</td>
<td>4.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Triton X-100 (0.1 mg/mg membrane protein)</td>
<td>4.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Triton X-100 and colchicine</td>
<td>3.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Verapamil (10 μM)</td>
<td>9.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Osmotically disrupted membranes</td>
<td>3.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Osmotically disrupted membranes + verapamil</td>
<td>8.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Under these conditions the Vₘₐₓ and Kₘ values for Mg-dATP hydrolysis were 6.7 μmol/mg P-glycoprotein/min and 1.1 mM, respectively.

Membranes were assayed in medium lacking sucrose, all other conditions being the same.

**Fig. 5.** pH dependence of CR1R12 plasma membrane ATPase activity. The pH profile of ATPase activity was measured at 37 °C in buffers containing 10 mM MgATP, 50 mM Tris succinate (at the appropriate pH), 1.0 mM EGTA, and 2 mM ouabain. pH was measured at 37 °C using a Sigma “Trizma” glass calomel combination electrode. 15–60 μg/ml CR1R12 plasma membranes was added to start the reaction. The activities given here were normalized to the weight of P-glycoprotein present in the membranes. Results are averages of quadruplicate determinations (±S.D.). The lines drawn are arbitrary. ○, 10 μM verapamil present; □, no verapamil.

**TABLE V**

**Nucleotide hydrolysis by P-glycoprotein**

CR1R12 plasma membranes were assayed at 50 μg/ml in buffers containing 5 mM magnesium nucleotide, 0.25 M sucrose, 0.1 mM EGTA, 2 mM ouabain, and 40 mM Tris-Cl, pH 7.4, at 37 °C. When added colchicine was at 120 μM, verapamil at 10 μM, and Triton X-100 was at 5 μg/ml. Here the activities were normalized to the amount of P-glycoprotein in the membranes.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Activity (μmol P/ mg P-glycoprotein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact membranes + colchicine + Triton X-100 + verapamil</td>
</tr>
<tr>
<td>MgATP</td>
<td>2.5 + 4.8 + 5.2 + 7.4</td>
</tr>
<tr>
<td>MgADP*</td>
<td>0 + 0 + 0 + 0</td>
</tr>
<tr>
<td>MgAMP</td>
<td>0 + 0 + 0 + 0</td>
</tr>
<tr>
<td>MgGTP</td>
<td>0.19 + 0.26 + 0.57 + 0.80</td>
</tr>
<tr>
<td>MgITP</td>
<td>0.40 + 0.61 + 0.87 + 1.5</td>
</tr>
</tbody>
</table>

* Kᵢ = 0.35 ± 0.15 mM (S.D.) versus MgATP, obtained from rate measurements at a range of MgADP and MgATP concentrations.

(not shown). Full inhibition of ATPase was achieved with the incorporation of 1.1 mol of NBD-Cl/mol of P-glycoprotein (calculated by extrapolation of data from Fig. 9). When plasma membranes were solubilized with 15 mM CHAPS and 20% (v/v) glycerol and the labeling experiment with [¹⁴C]NBD-Cl was repeated, a very similar pattern of labeling and inhibition was observed (data not shown).

In order to test the nature of the chemical adduct formed, CR1R12 plasma membranes were reacted with NBD-Cl as in Fig. 7A such that the P-glycoprotein ATPase was 50 and 100% inhibited. After removal of unreacted NBD-Cl by passage through a centrifuge column, the membranes were incubated with 1 mM DTT at pH 7.5 and 23 °C, and the ATPase activity was measured as a function of time of exposure to DTT. Up to 60 min there was no reactivation by DTT, clearly demonstrating that the adduct formed was not NBD-O-tyro-
centrifuge column. ATPase activity was measured in the buffer given verapamil. Plasma membranes (50 pg/ml) were preincubated for 10 min at 37 °C and protein/ml. The reaction was stopped by passage through a second protein concentration was adjusted to 0.23 µM, then 10 µM (final concentration as in above supplemented with 10 mM MgATP, 2 mM ouabain, and 10 µM NBD-Cl. CR1R12 plasma membranes (50 µg/ml) were preincubated for 10 min at 37 °C with 15 µM NBD-Cl in 0.25 M sucrose, 0.1 mM EGTA, and 40 mM Tris succinate at the appropriate pH. ATPase activity was then measured as above. The 100% activity was for membranes that were the same conditions as in membranes by NBD-Cl A, CR1R12 plasma membranes were first equilibrated with buffer (0.25 M sucrose, 0.1 mM EGTA, 40 mM Tris-Cl, pH 7.4) by passage through 1-ml Sephadex G-50 centrifuge columns (Penefsky, 1977) then incubated with NBD-Cl at 37 °C, for the various times given, at a final concentration of 55 µg of membrane protein/ml. The reaction was stopped by passage through a second centrifuge column. ATPase activity was measured in the buffer given above supplemented with 10 mM MgATP, 2 mM ouabain, and 10 µM verapamil. O, 0; □, 1.5; △, 7; Δ, 15; ⋄, 70, µM NBD-Cl. B, CR1R12 plasma membranes (50 µg/ml) were preincubated for 10 min at 37 °C with 15 µM NBD-Cl in 0.25 M sucrose, 0.1 mM EGTA, and 40 mM Tris succinate at the appropriate pH. ATPase activity was then measured as above. The 100% activity was for membranes that were at pH 7.4 in the absence of NBD-Cl.

FIG. 7. Inhibition of ATPase activity of CR1R12 plasma membranes by NBD-Cl. A, CR1R12 plasma membranes were first equilibrated with buffer (0.25 M sucrose, 0.1 mM EGTA, 40 mM Tris-Cl, pH 7.4) by passage through 1-ml Sephadex G-50 centrifuge columns (Penefsky, 1977) then incubated with NBD-Cl at 37 °C, for the various times given, at a final concentration of 55 µg of membrane protein/ml. The reaction was stopped by passage through a second centrifuge column. ATPase activity was measured in the buffer given above supplemented with 10 mM MgATP, 2 mM ouabain, and 10 µM verapamil. O, 0; □, 1.5; △, 7; Δ, 15; ⋄, 70, µM NBD-Cl. B, CR1R12 plasma membranes (50 µg/ml) were preincubated for 10 min at 37 °C with 15 µM NBD-Cl in 0.25 M sucrose, 0.1 mM EGTA, and 40 mM Tris succinate at the appropriate pH. ATPase activity was then measured as above. The 100% activity was for membranes that were at pH 7.4 in the absence of NBD-Cl.

FIG. 8. Covalent labeling of P-glycoprotein by [14C]NBD-Cl. A, kinetics of [14C]NBD-Cl reaction with CR1R12 plasma membranes. CR1R12 plasma membranes were first passed through a 1-ml Sephadex G-50 centrifuge column (Penefsky, 1977) to equilibrate them in 0.25 M sucrose, 40 mM Tris-Cl, pH 7.4, at 23 °C. P-glycoprotein concentration was adjusted to 0.23 µM, then 10 µM (final concentration) [14C]NBD-Cl was added. The reaction was stopped by passing 100-µl samples through Sephadex G-50 centrifuge columns at the times given in the figure. Thirty µl of each eluate was run on SDS-PAGE without DTT, and the gels were stained and fluorographed. B, reaction of [14C]NBD-Cl with AUXB1 plasma membranes under the same conditions as in A. C, pH dependence of the reaction of [14C]NBD-Cl with CR1R12 plasma membranes. CR1R12 plasma membranes (0.15 µg P-glycoprotein) were reacted with 10 µM [14C]NBD-Cl for 50 min at 23 °C. The buffers contained 50 mM Tris succinate at the appropriate pH values given in the figure, with other conditions as in A.

FIG. 9. [14C]NBD-Cl labeling of P-glycoprotein and inhibition of ATPase activity in CR1R12 plasma membranes. [14C]NBD-Cl was reacted with CR1R12 plasma membranes as in Fig. 5A. The P-glycoprotein band was cut out of the stained gels, end [14C]NBD-Cl incorporated into the protein was determined by counting radioactivity in gel slices as described under "Experimental Procedures." ATPase assays (with 5 µM verapamil present) were done on the same reacted samples. 140 kDa was used for the molecular mass of the P-glycoprotein polypeptide. The graph illustrates the inhibition of P-glycoprotein ATPase activity as a function of the stoichiometry of [14C]NBD-Cl covalently bound to P-glycoprotein.

A. CR1R12
B. AUXB1
C. CR1R12

Fig. 7. Inhibition of ATPase activity of CR1R12 plasma membranes by NBD-Cl. A, CR1R12 plasma membranes were first equilibrated with buffer (0.25 M sucrose, 0.1 mM EGTA, 40 mM Tris-Cl, pH 7.4) by passage through 1-ml Sephadex G-50 centrifuge columns (Penefsky, 1977) then incubated with NBD-Cl at 37 °C, for the various times given, at a final concentration of 55 µg of membrane protein/ml. The reaction was stopped by passage through a second centrifuge column. ATPase activity was measured in the buffer given above supplemented with 10 mM MgATP, 2 mM ouabain, and 10 µM verapamil. O, 0; □, 1.5; △, 7; Δ, 15; ⋄, 70, µM NBD-Cl. B, CR1R12 plasma membranes (50 µg/ml) were preincubated for 10 min at 37 °C with 15 µM NBD-Cl in 0.25 M sucrose, 0.1 mM EGTA, and 40 mM Tris succinate at the appropriate pH. ATPase activity was then measured as above. The 100% activity was for membranes that were at pH 7.4 in the absence of NBD-Cl.

Fig. 8. Covalent labeling of P-glycoprotein by [14C]NBD-Cl. A, kinetics of [14C]NBD-Cl reaction with CR1R12 plasma membranes. CR1R12 plasma membranes were first passed through a 1-ml Sephadex G-50 centrifuge column (Penefsky, 1977) to equilibrate them in 0.25 M sucrose, 40 mM Tris-Cl, pH 7.4, at 23 °C. P-glycoprotein concentration was adjusted to 0.23 µM, then 10 µM (final concentration) [14C]NBD-Cl was added. The reaction was stopped by passing 100-µl samples through Sephadex G-50 centrifuge columns at the times given in the figure. Thirty µl of each eluate was run on SDS-PAGE without DTT, and the gels were stained and fluorographed. B, reaction of [14C]NBD-Cl with AUXB1 plasma membranes under the same conditions as in A. C, pH dependence of the reaction of [14C]NBD-Cl with CR1R12 plasma membranes. CR1R12 plasma membranes (0.15 µg P-glycoprotein) were reacted with 10 µM [14C]NBD-Cl for 50 min at 23 °C. The buffers contained 50 mM Tris succinate at the appropriate pH values given in the figure, with other conditions as in A.

Inhibition of P-glycoprotein ATPase by a Range of Established ATPase Inhibitors

Table VI shows inhibition of P-glycoprotein ATPase by various well characterized ATPase inhibitors. The covalent inhibitors NEM and DCCD strongly inhibit several transport ATPases (Linnett and Beechey, 1979; Wallick et al., 1978; Panet and Selinger, 1970; Richards et al., 1977; Forte et al., 1975; Forgac, 1989). DCCD inhibited P-glycoprotein ATPase activity relatively weakly, thus there was no indication of catalytically important carboxylates using this reagent. NEM, on the other hand, proved to be a potent inhibitor, with 50% inhibition occurring at 1 µM reagent; the other tested sulfhydryl agent, HgCl₂, was equally effective. ATP was a good protecting agent against NEM inhibition (Table VI), and inclusion of 10 mM ATP gave nearly full protection against up to 50 µM NEM under the conditions used. These results indicate the presence of important cysteine(s) in P-glycoprotein that may well reside in the catalytic and or nucleotide-binding site(s) (see "Discussion").

Azide, a potent inhibitor of mitochondrial ATPase (Ebel and Lardy, 1975), was without effect on P-glycoprotein ATPase up to 10 mM and very little inhibition was observed at 50 mM NaF, which inhibits various phosphatases (Harper et al., 1978) and gastric H,K-ATPase (Forte et al., 1975), inhibited half-maximally at 3.3 mM. Human P-glycoprotein was reportedly not inhibited by 10 mM NaF (Sarkadi et al., 1992). The presence or absence of Al³⁺ could account for differences in degree of NaF inhibition seen (see e.g. Stur and Marquis, 1990, for a review of the inhibitory effects of fluoroulate complexes on various transport ATPases). In order to test this hypothesis the potent Al³⁺ chelator, deferoxamine, was added with the NaF, but no change in inhibition was seen, indicating that Al³⁺ was not interfering with the NaF inhibi-
P-glycoprotein ATPase Activity

Inhibition of ATPase activity of CR1R12 plasma membranes by established ATPase inhibitors

ATPase assays were performed in the presence of 10 μM verapamil, under conditions as described in Table III.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor conc. required for half-maximal inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCCD</td>
<td>200 μM*</td>
</tr>
<tr>
<td>NEM</td>
<td>1.3 μM*</td>
</tr>
<tr>
<td>NEM with 10 mM MgATP</td>
<td>190 μM*</td>
</tr>
<tr>
<td>NaF</td>
<td>0.28 μM*</td>
</tr>
<tr>
<td>NaF with 0.5 mM deoxoferric oxide</td>
<td>≥50 mM</td>
</tr>
<tr>
<td>AIF</td>
<td>61 μM*</td>
</tr>
<tr>
<td>NaN3</td>
<td>12 μM</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>0.44 mM*</td>
</tr>
<tr>
<td>Aurovertin B</td>
<td>64 μM</td>
</tr>
<tr>
<td>Efrapeptin</td>
<td>1.3 μM</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>0.40 μg/ml</td>
</tr>
<tr>
<td>Venturicidin</td>
<td>0.47 μg/ml</td>
</tr>
</tbody>
</table>

*Inhibitor concentrations were preincubated with 30 μg/ml of CR1R12 plasma membranes for 10 min in 0.25 mM sucrose, 0.1 mM EGTA, and 40 mM Tris-Cl, pH 7.4, at 37 °C. At the end of preincubation, samples were directly added to the ATPase assay medium.

**Table VI**

Inhibition of ATPase activity of CR1R12 plasma membranes by established ATPase inhibitors

The nonhydrolyzable ATP analog AMP-PNP has been used to unmask high affinity catalytic sites in ATPases (e.g., the mitochondrial ATPase, Kᵢ = 14 mM; Cross and Nalin, 1982), Kᵢ for competitive inhibition of P-glycoprotein ATPase was 0.44 mM, thus suggesting that AMP-PNP is acting by competing for the low affinity ATP-binding site. MgATP

The mitochondrial ATPase inhibitors efrapeptin, aurovertin B, oligomycin, and venturicidin (Linnett and Beechey, 1979) all inhibited P-glycoprotein ATPase (Table VI) at much higher concentrations than are required to inhibit the mitochondrial ATPase. All of these are hydrophobic compounds.

MesO was used to dissolve many of the drugs used in this study and is a useful tool in investigating the chemical mechanism of hydrolytic enzymes (de Meis, 1989; Al-Shawi and Senior, 1993). Up to 8% (v/v) MesO had no effect on P-glycoprotein ATPase activity, in hypotonically disrupted membranes, but above this concentration MesO was inhibitory. Half-maximal inhibition was seen at 20% (v/v) MesO. Up to 20% (v/v) MesO, inhibition was fully reversible upon dilution.

**DISCUSSION**

We obtained a highly multidrug-resistant Chinese hamster cell line (CR1R12) which constitutively expresses P-glycoprotein in amounts up to 32% by weight of total plasma membrane protein. This cell line grows well in suspension culture, providing a good source of plasma membranes enriched in P-glycoprotein for enzymatic and mechanistic studies, and potentially for purification and reconstitution studies. In this report we describe characteristics of membrane-bound P-glycoprotein ATPase activity.

Conditions were established to measure P-glycoprotein ATPase activity in plasma membrane preparations. EctoATPase was found to be negligible. Na,K-ATPase was eliminated by use of 2 mM ouabain and omission of sodium and potassium ions, and Ca-ATPase (which was very low) was eliminated by use of EGTA. ATPase activity showed a good correlation with P-glycoprotein content in different CR1R12 plasma membrane preparations (Fig. 3). CH₃C5 cells, from which CR1R12 was derived, showed proportionately less ATPase and P-glycoprotein content. AUB1 cells (the parental line) showed nearly zero ATPase and no P-glycoprotein. The ATPase activity was stimulated by verapamil, colchicine, vinblastine, and daunomycin in concentration ranges relevant to the transport of the corresponding drug or modulator. The specific ATPase activity, in the presence of verapamil, was calculated to be around 9 μmol/min/mg of P-glycoprotein at 37 °C and pH 7.4 (≈21 s⁻¹) which is similar to, but higher, than that recently measured for human P-glycoprotein expressed in insect (Sf9) cells (3–5 μmol/min/mg of P-glycoprotein; Sarkadi et al., 1992). The degree of phosphorylation and glycosylation of P-glycoprotein in our preparations has not been assayed, but we would point out that the protein is being expressed in its natural background. The turnover rate measured here is of similar order of magnitude to that of other transport ATPases, e.g. Na,K-ATPase and Ca-ATPase.

P-glycoprotein did not hydrolyze ADP, but ADP was a competitive inhibitor (Kᵢ = 0.35 mM). AMP also was not hydrolyzed, and it did not inhibit the ATPase activity, which may indicate that it did not bind, up to 5 mM. Km MgATP was 1.4 mM, and Km Mg-dATP was 1.1 mM with a rate (Vmax) 74% of ATP hydrolysis. MgATP was greatly preferred over CaATP as substrate, and magnesium cation did not inhibit up to 50 mM concentration. ITF and GTP were poor but real substrates. GAP and to a lesser extent ITP have been shown recently to support energized drug transport by human P-glycoprotein (Lelong et al., 1992). Mg-ATPase activity was maximal at around pH 7.3.

NBD-Cl was a potent inhibitor of the membrane ATPase activity, and [¹⁴C]NBD-Cl was used to covalently label P-glycoprotein. One-hundred % inactivation of ATPase activity corresponded to covalent incorporation of 1.1 mol of NBD-Cl/mol of P-glycoprotein. Labeling of AUB1 plasma membranes alongside CR1R12 membranes confirmed that the high ATPase activity in CR1R12 membranes was entirely ascribable to P-glycoprotein. ATP offered significant protection against NBD-Cl inactivation. Previously, NBD-Cl has been shown to inactivate ATPases by reaction with Tyr or Lys residues. Here the pH profile, kinetics of inactivation, and...
labeling of P-glycoprotein by NBD-Cl, and lack of reversibility with DTT are consistent with a single Lys being labeled. The protection by ATP and full inhibition by NBD-Cl may indicate that the critical lysine is present in a catalytic and/or nucleotide-binding site. Protein fragmentation and sequencing to locate the modified residue is possible at this point and will be pursued. Similarly, 2-azido-ATP was found to bind covalently on photolysis and to inhibit P-glycoprotein ATPase by 100% concomitant with incorporation of approximately 1 mol of 2-azido-ATP/mol of P-glycoprotein. Protein sequencing studies to characterize the ATP-binding site should also be feasible with this reagent.

NEM was found to be a potent inhibitor of P-glycoprotein ATPase activity, and ATP protected markedly against such inhibition, which could suggest a nucleotide-binding site location of the reacted cysteine(s). Such a reactive cysteine, namely residue 254 of the catalytic subunit A of vacuolar H+-ATPases, was found recently to be responsible for the inhibition of coated vesicle H+-ATPase by NEM (Feng and For- gac, 1992). This cysteine resides in the "homology A" (Walker et al., 1982) region of the nucleotide-binding site of vacuolar ATPase subunit A and, importantly, it is conserved in homology A regions of both nucleotide-binding sites of all hamster P-glycoprotein isoforms (Endicott et al., 1991). Therefore, it is reasonable to speculate that the inhibition of P-glycoprotein by NEM may be due to the modification of either or both homology A cysteines. In future work, using radioactive NEM, we intend to investigate the stoichiometry of covalent NEM labeling required for inhibition of ATPase activity and to determine the location of the covalent label in the protein sequence. The presence of a reactive cysteine within the nucleotide-binding sites may allow the development of purine analogs that contain sulfhydryl or alkylating groups as suicide inhibitors in order to overcome multidrug resistance in cancer chemotherapy. Such purine derivatives and suicide inhibitors have been used for the treatment of leukemia (e.g. 6-mercaptopurine and 6-thioguanine, Chabner and Myers, 1982). The sulfhydryl agent omeprazole (Im et al., 1985) is currently being used for the treatment of peptic ulcers by inhibiting the gastric H+-K+-ATPase.

Other ATPase inhibitors were tested, but were relatively ineffective, namely, azide, fluoride, AMP-PNP, aurovertin, efrapeptin, oligomycin, venturicidin, and DCCD, although complete or nearly complete inhibition could be obtained with most of these compounds if concentrations were raised to unusually high levels. The lack of potent inhibition by DCCD implies that carboxyl groups may not be critical for activity. At the present time the catalytic mechanism of P-glycoprotein ATPases is unknown. Whether both potential nucleotide-binding sites are required to interact to attain physiological catalysis rates, and whether both sites are in fact catalytic has not been defined. Moreover the binding affinity for substrates at the two potential nucleotide-binding sites is unknown. The lack of strong inhibition of ATPase activity by AMP-PNP suggests, however, that there is no tight (high affinity) catalytic or regulatory binding site for MgATP in P-glycoprotein. With the covalent inhibitors described in this work such questions regarding the catalytic mechanism can now be approached.

Vanadate did significantly inhibit ATPase activity, with 50% inactivation occurring at 12 μM, similar to that observed for inhibition of drug transport by P-glycoprotein (Horio et al., 1988) and for inhibition of ATPase in human P-glycoprotein (Sarkadi et al., 1992). This suggests a penta-coordinate phosphorus species occurs during catalysis, but the relatively low affinity for vanadate argues against involvement of a covalent phosphorylated enzyme intermediate. Additionally, it should be noted that the amino acid sequences of P-glycoproteins do not suggest a covalent intermediate occurs in the catalytic cycle because there is no homology to the P-type ATPases in the relevant segment of the nucleotide-binding domains. Fluoroluminate has also been used to investigate the catalytic mechanism of ATPase enzymes and could prove useful with P-glycoprotein since significant inhibition was seen here.

In the plasma membranes prepared here, there was an increase in ATPase activity of up to 2-fold in low concentrations of detergents, which, we suggest, is due to unmasking of latent active sites in the sealed plasma membrane vesicles. Detergents at higher concentrations apparently interfered with P-glycoprotein-drug interactions, considerably reducing the apparent activation of ATPase activity by drugs and modulators, a fact which has to be appreciated in P-glycoprotein purification and reconstitution studies.

In summary, we demonstrate the ATPase activity of P-glycoprotein in Chinese hamster ovary cell plasma membranes and present an initial characterization of catalysis. Our enriched membrane preparations from CR1R12 cells should now provide sufficient material for purification and reconstitution into proteoliposomes, and for more detailed characterization of catalysis. Also, these enriched membranes offer a simple enzymic assay to screen for non-cytotoxic therapeutic multidrug-reversal reagents that interact with P-glycoprotein and inhibit or activate the ATPase activity.

Acknowledgments—We thank Alex Guimbard, Tricia Pulvino, and Sumedha Bhagat for excellent technical assistance. Dr. Victor Ling graciously gave us the CH'C's and AUXR1 cells, and we thank him. We also thank Dr. Richard Cross for the generous gift of [a,P]2-azido-ATP.

REFERENCES
