Covalent Inhibitors of P-glycoprotein ATPase Activity*

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Verapamil-stimulated ATP hydrolysis by Chinese hamster P-glycoprotein in plasma membranes was shown to occur at a site(s) which is conformationally flexible and of relatively low affinity and specificity. Such properties distinguish P-glycoprotein from other transport ATPases. 8-Azido-ATP and 2-azido-ATP were excellent substrates, confirming that both analogs are suitable photoaffinity labels for investigating the catalytic site(s). Inactivation of ATPase activity occurred coincident with covalent incorporation of approximately two 8-azido-ATP/P-glycoprotein, with the incorporated analog distributed equally between N- and C-terminal halves of the molecule. N-Ethylmaleimide potently inactivated in an ATP-protected, dithiothreitol-irreversible manner, with maximal inactivation occurring coincident with incorporation of approximately two N-ethylmaleimide/P-glycoprotein. The critical catalytic site sulfhydryls were shown to be located equally in N- and C-terminal halves of the molecule. Sulfhydryl-substituted purines also gave substantial inhibition of P-glycoprotein ATPase activity, which was dithiothreitol reversible. The data provide guidelines for beginning investigation of catalytic site architecture by protein chemistry approaches.

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). Often 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) and Gotteeman (1993)). The amino acid sequences of P-glycoproteins exhibit strong homologies to nucleotide-binding domains. Each of the two predicted nucleotide-binding domains contains both of the “homology A” and “homology B” consensus sequences first identified by Walker et al. (1982) as diagnostic of nucleotide-binding sites.

It has been clearly established that, in multidrug-resistant cell lines, drug efflux is an energy-dependent process that requires ATP (Bradley et al., 1988; Gros et al., 1992). P-glycoprotein-containing plasma membrane vesicles exhibit ATP-hydrolysis-dependent drug accumulation (Horio et al., 1988; Kamimoto et al., 1989; Lelong et al., 1992; Doige and Sharom, 1992) and bind the ATP analog 8-azido-ATP (Cornwell et al., 1987; Schurr et al., 1989; Georges et al., 1991). Site-directed mutagenesis of either or both of the putative ATP-binding domains inhibited the drug-exclusion function (Azzaria et al., 1989; Boninson, 1990).

Recent reports have established that P-glycoprotein demonstrates substantial ATPase activity, comparable in turnover rate to that of other plasma-membrane ATP-dependent transport enzymes. Sarkadi et al. (1992) expressed human P-glycoprotein in S9 insect cells using a baculovirus vector and found substantial drug-stimulated ATPase activity in the plasma membranes, whereas the control “sham” expression vector did not elicit any such activity. Al-Shawi and Senior (1993) found that a Chinese hamster ovary cell line (CR1R12), selected for high resistance to colchicine, expressed high levels of P-glycoprotein and also high drug-stimulated ATPase activity in the plasma membranes. Doige et al. (1992) reported that detergent-solubilized Chinese hamster ovary P-glycoprotein showed drug-stimulated ATPase, and Ambudkar et al. (1992) and Sharom et al. (1993) showed that partially purified human or Chinese hamster ovary P-glycoprotein manifested drug-stimulated ATPase after detergent solubilization and reconstitution in proteoliposomes.

From the clinical standpoint, the demonstration that P-glycoprotein is an ATPase and shows ATP-dependent function is potentially an important development because characterization of enzyme catalysis could lead to rationally designed inhibitors. The use of covalent inhibitors of other transport ATPases has provided one of the major avenues of characterization of the structure and function of this class of enzymes. Our previous work showed that P-glycoprotein ATPase activity was inactivated potently by 4-chloro,7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). Inactivation of ATPase activity correlated linearly with covalent labeling, with 100% inactivation occurring at a stoichiometry of 1.1 mol of NBD-Cl/mol of P-glycoprotein (Al-Shawi and Senior, 1993), and the characteristics of inactivation suggested that there was a single reactive lysine residue located within one of the nucleotide-binding domains of P-glycoprotein. Whereas the use of purified P-glycoprotein preparations will indubitably prove valuable for pursuit of such studies in the future, successful clinical intervention will require inhibition or circumvention of P-glycoprotein in its natural membrane environment. Thus it is also important to establish the properties of P-glycoprotein in plasma membranes. Here, we show that sulfhydryl reagents are potentially valuable tools for
inactivating the ATPase activity of P-glycoprotein in plasma membranes, and we report studies of the compounds 8-azido-ATP, 2-azido-ATP, and fluorescein isothiocyanate as substrates or inactivators.

**EXPERIMENTAL PROCEDURES**

**Plasma Membrane Preparation**

Purified plasma membrane preparations were made from CR1R12 cells or the parent AUXB1 cells as described previously (Al-Shawi and Senior, 1993).

**Quantitation of P-glycoprotein Content of Plasma Membranes**

The P-glycoprotein content of plasma membranes was initially quantitated by laser densitometry of Coomassie Blue-stained SDS gels as described in Al-Shawi and Senior (1993). Two further procedures were used to verify the accuracy of this analysis. (a) We have recently purified Chinese hamster P-glycoprotein from CR1R12 cells to apparent homogeneity. The purified material shows a single band on SDS gels when visualized by Coomassie Blue or silver staining. We ran varying amounts of this material (as determined by BCA protein assay and amino acid analysis) on SDS gels, either alone or after mixing with varying amounts of purified plasma membranes, then quantitated the total amount of P-glycoprotein in the samples by laser densitometry as described above. The results showed a close agreement (±5%) between the amounts of P-glycoprotein actually run on the gels and that calculated by laser densitometry. (b) Multiple incremental amounts of purified P-glycoprotein (50–1,000 ng) and plasma membranes (50–500 ng) were adsorbed on nitrocellulose in a dot-blot apparatus, then immunoblotted with rabbit polyclonal anti-P-glycoprotein antibody (Al-Shawi and Senior, 1993) and developed using the Amersham ECL kit. Staining intensities of the multiple dots were compared by visual inspection, and it was seen that the amounts of P-glycoprotein in the plasma membranes as calculated from immunoblotted agreed very well with those determined by laser densitometry.

The P-glycoprotein content of the plasma membrane preparations used in this work ranged from 22 to 28% (w/w) of the total membrane protein.

**Quantitation of Radioactivity in Protein Bands in Gel Slices**

The protein band corresponding to P-glycoprotein was excised from SDS gels and counted as previously described (Al-Shawi and Senior, 1993).

The molecular size of P-glycoprotein polypeptide used for stoichiometry calculations was 140 kDa. It is pertinent to note that plasma membranes from the parent AUXB1 cells showed only minor Coomassie Blue staining in SDS gels at the position of P-glycoprotein (Al-Shawi and Senior, 1993), such that errors introduced into stoichiometry calculations by reaction of labeling reagents with proteins other than P-glycoprotein are negligible.

**Mild Trypsin Hydrolysis of P-glycoprotein in Plasma Membranes**

This was done as described by Georges et al. (1991). These workers demonstrated, using a series of monoclonal antibodies, that mild trypsin hydrolysis may be used to cleave membrane-bound Chinese hamster P-glycoprotein into two halves, an N-terminal glycosylated half with apparent molecular size on SDS gels of 100 kDa, and a C-terminal half with apparent molecular size of 65 kDa. We confirmed that the same results were obtained using CR1R12 plasma membranes.

Both trypsin fragments were clearly visible in Coomassie Blue-stained SDS gels, as would be predicted only the C-terminal fragment was seen to cross-react with the C494 monoclonal antibody, whereas both fragments cross-reacted with C219 monoclonal antibody (George et al., 1991). In order to quantitate the distribution of radioactive label between the two halves of P-glycoprotein which had been reacted with covalent labeling reagent, the fragments were excised from stained gels and radioactivity content was measured by scintillation counting.

**Assays of ATPase and Other Nucleoside Triphosphatase Activities**

In all assays of ATPase activity, the (low) ATPase activity due to the Na,K-ATPase was eliminated by inclusion of ouabain, and the (negligible) contribution due to Ca-ATPase was eliminated by EGTA. There was essentially zero ecto-ATPase or mitochondrial ATPase present (Al-Shawi and Senior, 1993).

**Covalent Labeling of P-glycoprotein in Plasma Membranes**

**N-Ethylmaleimide—** Plasma membranes were pre-equilibrated in buffer containing 0.25 x sucrose, 0.1 mM EGTA, 20 mM Tris, pH 7.4, by passage through a 1-m1 centrifuge column containing Sephadex G-50 (Pharmacia) and adjusted to a concentration of 70–100 mg of membrane protein/ml, then reacted with various concentrations of [14C]N-ethylmaleimide (NEM) (see figure legends) for 10 min at 23 °C in the presence or absence of 10 mM Mg2+.

**Fluorescein Isothiocyanate—** Plasma membranes were pre-equilibrated in buffer by passage through centrifuge columns as above and adjusted to a protein concentration of 1.5 mg/ml. Photolabeling was carried out in 290-μl volumes in wells of 96-well microtiter plates at 23 °C in a humid air enclosure. [3H]8-Azido-ATP was added at various concentrations (see figure legends), and the samples were illuminated by a UV-2S Minilight 250-nm lamp placed directly over the plate. At time intervals, the samples were diluted with the addition of 30 μl of 50 mM Tris, pH 7.4, and 100-μl samples were passed through 1-md centrifuge columns to remove unreacted 8-azido-ATP.

**Fluorescein Isothiocyanate—** Plasma membranes were suspended at 125 μg/ml in 40 mM Tris-Cl, 1 mM EGTA, pH 9.0, at 37 °C, and fluorescein isothiocyanate isomer 1 (FITC), freshly dissolved in dimethyl sulfoxide, was added at varying concentrations. After 6 min of reaction, samples were removed and either assayed directly for ATPase activities, or added to SDS solution for gel electrophoresis. We showed previously that low concentrations of dimethyl sulfoxide (as added here with the FITC) do not inhibit P-glycoprotein ATPase activity (Al-Shawi and Senior, 1993).

**Routine Procedures**

SDS gel electrophoresis, immunoblotting, and protein assays were all performed as previously described in Al-Shawi and Senior (1993).

**Immunoprecipitation of P-glycoprotein from solubilized plasma membranes**

Immunoprecipitation of P-glycoprotein from solubilized plasma membranes was done essentially as described by Georges et al. (1991).

**Other Materials—** [3H]8-Azido-ATP was from Research Products International. [39P]8-Azido-ATP was from ICN. [14C]NEM was from Du Pont-New England Nuclear. Tissue culture materials were from Life Technologies Inc. and general chemicals from Sigma. C219 antibody was from Centocor Diagnostics and C494 antibody was from Signet Laboratories.

**RESULTS**

As emphasized in the Introduction there are important reasons to establish the characteristics of the catalytic sites of both membrane-bound and purified P-glycoprotein. In this work we have studied membrane-bound P-glycoprotein using plasma membranes purified from Chinese hamster ovary CR1R12 cells as source material. These plasma membranes are considerably enriched in P-glycoprotein (Al-Shawi and Senior, 1993).

**P-glycoprotein Isoform in CR1R12 Plasma Membranes**

Western blots showed that the P-glycoprotein band in plasma membranes from CR1R12 cells cross-reacted strongly

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which is the isoform causing multidrug-resistance in human ATP. The value of has been shown to cause amplification of the same isoform (Kartner et al., 1984), as was used for generation of CR1R12 from CHRC5 line (itself originally derived from the Am1 cell line) contains cell line was derived from the CHRC5 cell line. The CHRC5 cell was derived from the CR1R12 plasma membranes, although we cannot rule out the presence of minor amounts of other isoforms. The CR1R12 cell line was derived from the CHRC5 cell line. The CHRC5 cell line (itself originally derived from the AUXB1 cell line) contains primarily P-glycoprotein isoform-1 and further selection on colchicine, as was used for generation of CR1R12 from CHRC5, has been shown to cause amplification of the same isoform (Kartner et al., 1984; Georges et al., 1991). Therefore our findings are fully consistent with previous work. Chinese hamster P-glycoprotein isoform-1 is equivalent to human mdr1 isoform, which is the isoform causing multidrug-resistance in human cancer.

Further Characterization of the Catalytic Activity of P-glycoprotein Using Substrate Analogs

Fig. 1 shows the hydrolysis of 8-azido-ATP and 2-azido-ATP; Table I summarizes the kinetic parameters derived from these curves and includes also corresponding data for 2-dATP and ATP. The value of $k_{cat}/K_m$ is seen to be almost the same for all four of these substrates, and is relatively low compared to other transport ATPases. Because 8-azido-ATP and 2-azido-ATP usually adopt predominantly the syn- and anti-conformations, respectively, in solution (Czarnecki, 1984), the results suggest that the P-glycoprotein catalytic site(s) is unusually conformationally flexible. From the data of Table I, the catalytic site(s) is also seen to be of low affinity and relatively low specificity for the base and ribose moieties. These data, together with the lack of strong inhibition by the analog AMPPNP, provide evidence that there is no high affinity ATP-binding site involved directly in catalysis, a feature that distinguishes P-glycoprotein from other transport ATPases such as Na,K-ATPase and F1,F0-ATPase, and has interesting mechanistic implications. The values of $K_m$, ATP = 1.4 mM and $K_m$, ADP = 0.35 mM imply that in vivo the ATPase activity could well be responsive to cellular levels of these nucleotides.

![Graph showing hydrolysis of 8-azido-ATP and 2-azido-ATP by P-glycoprotein](image)

**Table I**

Specificity of P-glycoprotein for Mg nucleotide substrates and inhibitors

| Nucleotide | $V_{max}$ | $K_m$ | $k_{cat}/K_m$ |
|------------|-----------|-------|--------------|
| ATP        | 9.0       | 1.4   | $1.5 \times 10^4$ |
| 2'-dATP    | 6.7       | 1.1   | $1.4 \times 10^4$ |
| 2-Azido-ATP| 1.8       | 0.37  | $1.1 \times 10^4$ |
| 8-Azido-ATP| 8.6       | 0.50  | $1.6 \times 10^4$ |
| GTP        | 0.97      | ND*   | ND*          |
| TTP        | 1.8e      | ND*   | ND*          |
| ADP        | 0         | $K_m = 0.35$ |
| AMP       | 0         | $K_m = 4.4$ |

* The $K_m$ values for GTP and TTP were high (>3 mM) and could not be calculated accurately. Hence, the $V_{max}$ values may also be underestimated.
* ND, not determined.

**Inactivation and Photoaffinity Labeling of P-glycoprotein by 8-Azido-ATP and 2-Azido-ATP**

The data provided above demonstrate that 8-azido-ATP and 2-azido-ATP are substrates for hydrolysis by P-glycoprotein. Labeling of P-glycoprotein by 8-azido-ATP has been accomplished previously as described in the Introduction, but it has not been established whether this analog is actually a substrate and no measurements of relationship of inactivation of ATPase activity to stoichiometry of labeling have been reported. Fig. 2 demonstrates that [a-$32P$]8-azido-ATP labels P-glycoprotein when incubated with CR1R12 plasma membranes and then photoactivated. The parent drug-sensitive AUXB1 cell line, which contains only low amounts of P-glycoprotein in plasma membranes (Kartner et al., 1985; Al-Shawi and Senior, 1993), showed insignificant labeling at the position of P-glycoprotein on gels. Fig. 3 shows the degree of inactivation of ATPase activity plotted versus the stoichiometry of covalent incorporation of 8-azido-ATP. The data show that maximal inactivation occurs with covalent incorporation of approximately 2 mol of 8-azido-ATP/mol of P-glycoprotein. Similar results were seen in the absence or presence of verapamil (10 µM).

Georges et al. (1991) showed previously using a mild trypsin digestion procedure that in P-glycoprotein labeled at a low stoichiometry with [$32P$]8-azido-ATP, the radioactivity was seen by autoradiography to be approximately equally distributed between N- and C-terminal halves of the molecule. This work suggested that both nucleotide-binding domains were reacting with 8-azido-ATP. Here we determined that in samples of P-glycoprotein that were labeled to the extent of 0.6 and 1.4 mol/mg by [a-$32P$]8-azido-ATP, the distribution of radioactivity between the two halves as determined by direct counting of bands excised from gels was: N-terminal, 52%; C-terminal, 48% (mean of four experiments at each labeling stoichiometry). Our data suggest that at substantial levels of labeling, the two P-glycoprotein nucleotide-binding sites became labeled in equal proportion.

Previously we reported that radioactive 2-azido-ATP inactivated P-glycoprotein ATPase activity on photoactivation, concomitant with incorporation of approximately 1 mol of 2-azido-ATP/mol of P-glycoprotein (Al-Shawi and Senior, 1993). In that work, we used [$\beta,\gamma$-$32P$]2-azido-ATP. Our new finding that 2-azido-ATP is actually a good substrate for hydrolysis by P-glycoprotein (Fig. 1, Table I) requires re-evaluation of that data. Whereas previously we had calculated the labeling stoichiometry based on the initial specific radioactivity of the

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ATP, inactivated P-glycoprotein by covalently reacting at membranes with $[^{32}P]8$-azido-ATP. Covalent labeling was done at pH 7.4 as described under "Experimental Procedures." The concentration of $[^{32}P]8$-azido-ATP was 1.5 mM. Forty μl (=15 μg of protein) of the labeled sample was run on 10% SDS gels without DTT and the gels were subjected to autoradiography. The times of incubation are shown above the lanes. CR1R12 refers to the highly multidrug-resistant cells; AUXB1 are the parental drug-sensitive cells. When the experiment was done at pH 8.5, the same results were obtained.

2-azido-ATP, it is now evident that up to half of the $^{32}P$ would be released due to hydrolysis of the γ-phosphate (see Czarnecki (1984)). Thus, it now appears that 2-azido-ATP, like 8-azido-ATP, inactivated P-glycoprotein by covalently reacting at approximately two sites. This question may be further addressed in future studies using $[^{32}P]$-labeled 2-azido-ATP.

**Inactivation and Covalent Labeling of P-glycoprotein with N-Ethylmaleimide**

Fig. 4 shows the potent inactivation of P-glycoprotein by NEM and the protection afforded by 10 mM MgATP present during the reaction. This data suggests that there is a critical sulfhydryl group(s) present within the catalytic site(s) which is reactive. The pH dependence of inactivation was consistent with the reaction with NEM occurring at a cysteine residue(s). The inactivation of P-glycoprotein ATPase activity by NEM was completely nonreversible by DTT. It was also found that NEM inactivated the hydrolysis of 8-azido-ATP and 2-azido-ATP with the same characteristics as for inactivation of ATPase.

Fig. 5 shows that NEM labeled P-glycoprotein heavily in CR1R12 cell plasma membranes. When plasma membranes from the parent drug-sensitive AUXB1 cell line were studied, no labeling was seen at the position equivalent in mobility to P-glycoprotein (data not shown). Fig. 6 shows the degree of inactivation of P-glycoprotein ATPase activity plotted against the stoichiometry of covalent labeling of P-glycoprotein by $[^{14}C]$NEM. Similar results were seen in the presence or absence of verapamil (10 μM). The dotted line in Fig. 6 is a linear least squares fit to all the data and implies that complete inhibition occurred at $\sim 1.6$ sites reacted. The solid line is a nonlinear regression fit (as detailed in Fig. 6, legend) which also implies that there are around two total sites reacting and essential for catalysis. A conservative interpretation of the data is that inactivation is associated with reaction of only a small number of residues and that it should be feasible to characterize the critical reactive group(s) using protein fragmentation and sequencing procedures.

$[^{14}C]$NEM-labeled P-glycoprotein was fragmented into N- and C-terminal halves by mild trypsin proteolysis as described under "Experimental Procedures" and run on SDS gels. After excision and counting of the bands, the radioactivity was found to be distributed equally between the two fragments as follows: N-terminal, 56%; C-terminal, 44% (mean of four experiments). We should also note here that in order to see potent inactivation by NEM, effective removal of DIT by passage through centrifuge columns was required, as described under "Experimental Procedures." Simple dilution of the P-glycoprotein-containing samples into assay or reaction buffer was not sufficient, implying protection by DTT. An analogous situation was seen when the inactivation of P-glycoprotein ATPase activity plotted against the stoichiometry of covalent labeling of P-glycoprotein by $[^{32}P]8$-azido-ATP was carried out as described in the legend to Fig. 2, except that initially 0.5 μM analog was added, followed by three further additions of 0.5 μM analog at 30-min intervals for a total incubation period of 120 min. Samples were withdrawn at intervals and assayed for ATPase activity (as described under "Experimental Procedures") or subjected to gel electrophoresis (as described in the legend to Fig. 2). The gels were fixed with acetic acid containing Coomassie Blue stain, destained briefly, then the P-glycoprotein band was cut out and counted as described under "Experimental Procedures." The solid line is the best nonlinear regression fit to the data using the theoretical framework of Tsou (1962) for analyzing chemical modification experiments as discussed in Cornish-Bowden (1979). The simplest case was considered in which there are λ groups on each P-glycoprotein monomer that react at the same rate and that μ of these groups are essential for catalytic activity. Then $\alpha = (1 - \gamma \lambda)^\mu$, where $\alpha$ is the fractional activity remaining when γ groups in the monomer have been modified. The best fit was obtained with $\lambda = 2.0$ (total sites modified in each P-glycoprotein monomer) and $\mu = 2.0$ (number of the modified sites essential for catalysis).
then the reaction with radioactive NEM was initiated.

Carried out, the membranes were passed through centrifuge columns, conditions were as described under "Experimental Procedures." Conditions were the same as above except that a preincubation in 50 mM plasma membranes by ["4ClI" ethylmaleimide. The reaction con-

['TINEM (2 μM) was added to start the reaction and the incubation was continued for the times shown. 30-μl samples were run on 7–25% gradient SDS gels, and the gels were subjected to fluorography. Panel B, conditions were the same as above except that a preincubation in 50 mM MgATP plus 1 μM nonradioactive NEM for 20 min ("blocking") was carried out, the membranes were passed through centrifuge columns, then the reaction with radioactive NEM was initiated.

**Fig. 5. Covalent labeling of P-glycoprotein (PGP) in CR1R12 plasma membranes by ["4ClI"-ethylmaleimide.** The reaction conditions were as described under "Experimental Procedures." Panel A, ["4ClI"NEM (2 μM) was added to start the reaction and the incubation was continued for the times shown. 30-μl samples were run on 7–25% gradient SDS gels, and the gels were subjected to fluorography. Panel B, conditions were the same as above except that a preincubation in 50 mM MgATP plus 1 μM nonradioactive NEM for 20 min ("blocking") was carried out, the membranes were passed through centrifuge columns, then the reaction with radioactive NEM was initiated.

**TOP**

**PGP**

**FRONT**

**Fig. 6. Covalent labeling and inactivation of ATPase activity of P-glycoprotein (PGP) by ["4ClI"NEM in plasma membranes.** Conditions were as described in the legend to Fig. 5, panel A, except that varying concentrations of radioactive NEM (2–20 μM) were added to achieve different levels of inactivation of ATPase activity. At the end of the reaction, samples were assayed for ATPase or run on SDS gels as in Fig. 5. The P-glycoprotein band was excised from stained gels for determination of incorporated radioactivity. The dotted line is a linear least squares fit to all the data points; the solid line is a nonlinear regression fit, derived as described in the legend to Fig. 3. The best fit was obtained with k = 2.2 (total sites modified in each P-glycoprotein monomer) and μ = 1.6 (number of sites modified that are essential for catalysis).

For protection of clathrin-coated vesicle ATPase by cystine against NEM inactivation (Feng and Forgac, 1992).

**Inhibition of P-glycoprotein ATPase Activity by Sulphhydryl-substituted Purine Compounds**

**Reversible Inhibition—**Table II shows the concentrations of a range of sulphhydryl-substituted purine compounds required for 50% inhibition of P-glycoprotein ATPase activity. Several of these compounds have been used in cancer chemotherapy (Elion, 1992). In Table II the ATPase assays were conducted for 5–10 min at 37 °C, pH 7.4, in the presence of the purine compounds. We have called this inhibition "reversible" because if the membranes were preincubated in the same buffer lacking MgATP for 5–10 min, then passed through centrifuge columns and assayed for ATPase activity, there was no evident inhibition of ATPase activity. This type of inhibition was further explored in the case of 6-mercaptopurine by measuring ATPase rates in the presence of a range of 6-mercaptopurine and MgATP concentrations. It was determined that K_M ATP apparent did not vary, whereas V_max apparent did, showing the inhibition to be noncompetitive, with calculated K_i of 9 μM for 6-mercaptopurine.

**Nonreversible Inhibition—When CR1R12 plasma membranes were incubated with higher concentrations of 6-mercaptopurine in buffer lacking MgATP for 10 min at 37 °C as above, then passed through centrifuge columns to remove free and loosely bound 6-mercaptopurine before assaying for ATPase activity, a persistent ("nonreversible") inhibition of ATPase activity occurred. 50% inhibition occurred with 1 mM 6-mercaptopurine under these conditions. Incubation of the inhibited membranes with 1 mM DTT for 60 min at 23 °C caused complete reactivation of the ATPase, suggesting that the inhibition was due to covalent reaction of 6-mercaptopurine with one or more sulphhydryl groups. The reaction with 6-mercaptopurine did not protect against DTT-irreversible NEM inhibition.

We attempted to determine the stoichiometry of incorporation of 6-mercaptopurine into P-glycoprotein in CR1R12 plasma membranes and its relationship to nonreversible ATPase inhibition, following the procedures described above for 8-azido-ATP and NEM. SDS-gel electrophoresis and fluorography showed that major incorporation of radioactivity into the P-glycoprotein band occurred. However, it was apparent that there was significant loss of P-glycoprotein bound radioactivity during the course of the analysis and so the labeling stoichiometry could not be determined with confidence.

These experiments showed therefore that there are critical sulphhydryl group(s) in P-glycoprotein, probably in addition to the ones labeled by NEM, which react with sulphhydryl-substituted purines at higher concentrations.

**Inhibition of P-glycoprotein ATPase Activity by Fluorescein Isothiocyanate**

FITC has proved very useful in characterizing the catalytic site of Na,K-ATPase (Karlish, 1980; Farley et al., 1984) and gastric ATPase (Farley and Faller, 1985). In both cases specific reaction with a lysine residue was seen. Fig. 7 shows that while FITC inhibited the Na,K-ATPase activity of CR1R12 plasma membranes potently, in agreement with expected results from previous work (Karlish, 1980), it was only a weak inhibitor of.
Covalent Inhibitors of P-glycoprotein

P-glycoprotein ATPase. The reacted membranes were analyzed by SDS-gel electrophoresis and fluorescence of the bands was visualized by excitation with a long UV lamp. P-glycoprotein was apparently not significantly labeled by FITC. The α-subunit of the Na,K-ATPase was barely visible as a fluorescent band running at 100 kDa (the Na,K-ATPase content of these membranes is known to be low from previous work).

While these results show that FITC is not a useful covalent probe for P-glycoprotein catalytic site(s), they are of value in showing that FITC may be used in studies using plasma membrane preparations to block the Na,K-ATPase nucleotide-binding sites while leaving P-glycoprotein sites relatively unreacted.

**DISCUSSION**

In previous work on Chinese hamster ovary P-glycoprotein (Al-Shawi and Senior, 1993) we reported the generation of an enriched source material for experimentation, namely the plasma membrane preparation obtained from CR1R12 cells, which contains large amounts of P-glycoprotein, and we described an initial characterization of the P-glycoprotein ATPase activity. Here we have extended the characterization of the catalytic sites by use of a range of different substrates, and we have shown that several covalent inactivating reagents are potentially useful probes for establishing the architecture of the catalytic site(s) by protein chemistry techniques. The long-term benefits of this approach could be the rational design of catalytic site-binding agents to inactivate P-glycoprotein in vivo.

As noted under "Results," ATP, Z'-dATP, 8-azido-ATP, and 2-azido-ATP were equally good substrates for hydrolysis, showing that nucleotide substrate binding and hydrolysis on P-glycoprotein occurs at a relatively loose, nonspecific, and conformationally flexible site or sites. Both 8-azido-ATP and 2-azido-ATP apparently bound and reacted with catalytic sites. Thus, each of these two photoaffinity reagents may be used to probe the nucleotide-binding sites. The use of both probes is likely to be complementary, since as noted above, they are known to adopt different conformations in solution, and may therefore react with different subdomains of the nucleotide-binding sites.

In mitochondrial F1-ATPase, 8-azido-ATP reacts with Tyr-311 of the catalytic site (Hollemans et al., 1983), whereas 2-azido-ATP reacts with Tyr-345 (Cross et al., 1987). Use of these reagents could also reveal whether nucleotide binding to the two different predicted nucleotide sites on each P-glycoprotein molecule is stereochemically equivalent or nonequivalent.

The experiments with N-ethylmaleimide demonstrate the presence of critical sulfhydryl groups in P-glycoprotein, most likely located within the catalytic sites, since strong protection from inactivation was afforded by MgATP. Also, NEM reacted at approximately two sites, and mild trypsin proteolysis experiments revealed equal distribution of radioactive NEM between the N- and C-terminal halves of labeled P-glycoprotein. The homology A sequences in the two predicted nucleotide-binding domains of Chinese hamster P-glycoprotein (which are, respectively, GNSGCGKS and GSSCGCGKS) both contain a cysteine residue, and the same sequences are present in human mdr1. Moreover there is no other cysteine present in the cytoplasmic domain of the N-terminal half of Chinese hamster P-glycoprotein isoform-1. Protein sequencing of NEM-labeled P-glycoprotein is now feasible and will be pursued to locate the reactive cysteines.

We now work with the reagent NBD-Cl that had shown that inactivation occurred rapidly, and that a lysine residue was a likely target of the NBD-Cl. Mild trypsin proteolysis of [14C]NBD-Cl-labeled P-glycoprotein in plasma membranes has now demonstrated that the bulk of the radioactivity is located in the C-terminal half after fragmentation and protein chemical identification of the labeled residue is in progress. The reagent fluorescein isothiocyanate is also known to react specifically with a lysine residue in the catalytic site of Na,K-ATPase (Karlsh, 1980; Farley et al., 1984) and gastric ATPase (Farley and Faller, 1985). However, FITC was found here to be a relatively weak inhibitor of P-glycoprotein ATPase activity. Other affinity reagents which react with lysine residues in nucleotide-binding proteins are the pyridoxal-substituted adenosine nucleotides (Tamura et al., 1986; Rao et al., 1988) and the fluorosulfonylbenzoyl-substituted purine ribosides (Zoller and Taylor, 1979; Colman, 1990) and these may be useful probes of P-glycoprotein.

In conclusion, this work provides guidelines for exploration of the catalytic sites of P-glycoprotein by protein chemical methodology, and the identification of potential target residues for inhibitors.

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