Both P-glycoprotein Nucleotide-binding Sites Are Catalytically Active*

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The technique of vanadate trapping of nucleotide was used to study catalytic sites of P-glycoprotein (Pgp) in plasma membranes from multidrug-resistant Chinese hamster ovary cells. Vanadate trapping of Mg- or Co-8-azido-nucleotide (1 mol/mol of Pgp) caused complete inhibition of Pgp ATPase activity, with reactivation rates at 37 °C of 1.4 × 10^{-3} s^{-1} (t_{1/2} = 8 min) or 3.3 × 10^{-4} s^{-1} (t_{1/2} = 35 min), respectively. UV irradiation of the inhibited Pgp yielded permanent inactivation of ATPase activity and specific photolabeling of Pgp. Mild trypsin digestion showed that the two nucleotide sites were labeled in equal proportion. The results show that both nucleotide sites in Pgp are capable of nucleotide hydrolysis, that vanadate trapping of nucleotide at either site completely prevents hydrolysis at both sites, and that vanadate trapping of nucleotide in the N- or C-terminal nucleotide site occurs non-selectively. A minimal scheme is presented to explain inhibition by vanadate trapping of nucleotide and to describe the normal catalytic pathway. The inhibited Pgp-Mg-nucleotide-vanadate complex is probably an analog of the catalytic transition state, implying that when one nucleotide site assumes the catalytic transition state conformation the other site cannot do so and suggesting that the two sites may alternate in catalysis.

P-glycoprotein (Pgp),¹ also called multidrug-resistance protein, is involved in resistance of tumors to a variety of drugs and appears to be responsible for many failures of cancer chemotherapy (reviewed in Endicott and Ling (1989), Gottesman and Pastan (1993), and Gros and Buschman (1993)). Pgp is a plasma membrane protein that reduces intracellular accumulation of drugs by transporting them out of the cell in an ATP hydrolysis-dependent process (Ruetz and Gros, 1994) (reviewed in Shapiro and Ling (1995) and Sharom (1995)). Coupling between catalytic sites for ATP hydrolysis on the cytoplasmic surface and drug-binding/transport sites in the membrane is also apparent from the drug stimulation of ATP hydrolysis seen in plasma membrane preparations (Sarkadi et al., 1992; Al-Shawi and Senior, 1993), partially purified and reconstituted Pgp (Ambudkar et al., 1992; Sharom et al., 1993), and purified, reconstituted Pgp (Shapiro and Ling, 1994; Urbatsch et al., 1994; Sharom et al., 1995). The amino acid sequence of Pgp reveals a tandemly duplicated molecule, each half containing one cytoplasmically sided nucleotide-binding site, as diagnosed by the presence of both “homology A” and “homology B” consensus sequences (Walker et al., 1982). A major goal of current research is to determine the mode of operation of these two nucleotide-binding sites in the mechanism of action of Pgp. From the data available it has not yet been possible to determine the number of sites that are catalytically active in Pgp. There is a single relatively high Km(MgATP) around 1 mM, and ATP hydrolysis follows simple monophasic Michaelis-Menten kinetics. MgADP and MgAMP-PNP behave as classical competitive inhibitors with K_i values of 0.70 and 0.35 mM, respectively (Al-Shawi and Senior, 1993; Urbatsch et al., 1994). With 8-azido-ATP, which is an excellent substrate for hydrolysis by Pgp, photoinactivation of the Pgp ATPase occurred coincident with covalent incorporation of approximately 2 mol of 8-azido-[γ-32P]ATP/mol of Pgp, with the incorporated analog distributed equally between N- and C-terminal halves of the molecule. These data indicated that both nucleotide-binding domains were capable of binding 8-azido-ATP (Georges et al., 1991; Al-Shawi et al., 1994). The inhibitor N-ethylmaleimide reacted at two sites, with the critical sulfhydryls located equally in N- and C-terminal halves of the Pgp, whereas NBD-CI, another catalytic site covalent inhibitor, gave full inactivation at ~1 mol of MgATP per mol of Pgp, with reaction occurring predominantly in the C-terminal half (Al-Shawi and Senior, 1993; Al-Shawi et al., 1994). Since MgATP afforded full protection against inhibition by both N-ethylmaleimide and NBD-CI, it appeared that both nucleotide sites could bind MgATP and that inhibition at the C-terminal site alone gave 100% inhibition of ATPase activity.

Introduction of point mutations into either or both of the nucleotide-binding domains by site-directed mutagenesis inhibited the drug exclusion function of Pgp expressed in mammalian cells (Azzaria et al., 1989; Roninson, 1992). Loo and Clarke (1994) expressed each half of the Pgp molecule separately in Sf9 cells and found that each half-molecule displayed significant ATPase activity. This experiment showed that each nucleotide site has the potential capability for ATP hydrolysis. The state of oligomerization (homodimer? monomer?) of the expressed half-molecules of Pgp was not determined. Drug stimulation of the Pgp ATPase was apparent only in intact Pgp or when both half-molecules were expressed contemporaneously in the same cell, and Loo and Clarke concluded that coupling of ATPase activity to drug binding/transport requires interaction of both halves of the Pgp.

We have recently found that vanadate trapping of nucleotide at catalytic sites of Pgp induces stable inhibition of ATPase activity (Urbatsch et al., 1995). The nucleoside diphosphate was the trapped species whether vanadate inhibition was induced with MgATP or MgADP, and manganese or cobalt were also effective as metal cofactors. Complete inactivation of ATPase was achieved with trapping of 1 mol of MgADP/mol of Pgp, and full reactivation of ATPase correlated with release of a single mol of trapped Mg-[γ-32P]ADP per mol of Pgp. It has

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¹The abbreviations used are: Pgp, P-glycoprotein; AMPNP, adenosine 5‘-(β,γ-mimino)triphosphosphate; NBD-CI, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; V₅, orthovanadate.
Vanadate-induced inhibition of the ATPase catalytic sites—ATPase activity in the eluates. Unbound ligands by centrifuge column elution and assay of Pgp cleotide, divalent cation, and vanadate, followed by removal of technique involved preincubation of plasma membranes with nucleotide, divalent cation, and vanadate, followed by removal of unbound ligands by centrifuge column elution and assay of Pgp ATPase activity in the eluates.

Vanadate-induced inhibition of Pgp—This was done as described in Urbatsch et al. (1995). Briefly, plasma membranes (10 μg) were incubated with 200 μM vanadate, 200 μM nucleotide, 3 mM MgSO4 or CoSO4, 10 μM verapamil, 2 mM ouabain, 0.1 mM EGTA, and 40 mM Tris-HCl, pH 7.4, in a total volume of 100 μl for 20 min at 37°C. (Variations of these conditions are detailed in the figures.) The incubations were started by addition of membranes and stopped by passage of the 100-μl samples through centrifuge columns consisting of 1 ml of Sephadex G-50 (fine) topped with a 10-mm layer of Dowex AG1-X8 (Bio-Rad) (Penefsky, 1977; Wolodko et al., 1983) equilibrated with 0.1 mM EGTA, 40 mM Tris-HCl, pH 7.4, at 23°C. Control experiments using [α-32P]ATP showed that <0.002% of the applied nucleotide eluted from the columns in the absence of membranes.

Preparation of Plasma Membranes—Plasma membranes were prepared from the multidrug-resistant Chinese hamster ovary cell line CR1R12 as described (Al-Shawi and Senior, 1993; Urbatsch et al., 1995). The membranes contained from 15 to 24% w/w of Pgp as a fraction of total membrane protein.

Assay of Pgp ATPase Activity—Pgp ATPase activity was measured as described in Urbatsch et al. (1995). The Pgp ATPase activity was around 1.3 μmol of ATP hydrolyzed per min per mg of membrane protein.

EXPERIMENTAL PROCEDURES

Preparation of Plasma Membranes—Plasma membranes were prepared from the multidrug-resistant Chinese hamster ovary cell line CR1R12 as described (Al-Shawi and Senior, 1993; Urbatsch et al., 1995). The membranes contained from 15 to 24% w/w of Pgp as a fraction of total membrane protein.

Materials—[α-32P]ATP was from ICN. Tissue culture materials were from Life Technologies, Inc. C219 anti-Pgp monoclonal antibody was from Signet Laboratories.

RESULTS

Vanadate Trapping of 8-Azido-adenine Nucleotide in Pgp Catalytic Sites—Vanadate-induced inhibition of the ATPase activity of Pgp in plasma membranes from multidrug-resistant Chinese hamster ovary CR1R12 cells was carried out as described under “Experimental Procedures.” Briefly, the technique involved preincubation of plasma membranes with nucleotide, divalent cation, and vanadate, followed by removal of unbound ligands by centrifuge column elution and assay of Pgp ATPase activity in the eluates.

With 200 μM Mg-8-azido-ATP and Co-8-azido-ATP the degree of inhibition was 85 and 95%, respectively. Similar results were seen with Mg-8-azido-ADP and Co-8-azido-ADP. Reactivation of Pgp ATPase activity occurred when the centrifuge column eluates were incubated at 37°C. Fig. 1A shows the reactivation after inhibition in the presence of 200 μM Mg-8-azido-ATP or Mg-8-azido-ADP. The time courses for reactivation in the two cases were identical (t1/2 = 8 min; kapp = 1.4 × 10⁻⁴ s⁻¹). Fig. 1B shows the reactivation after inhibition in the presence of the corresponding Co-nucleotides. Here, the time courses were appreciably slower but were again the same for the two cases (t1/2 = 35 min; kapp = 3.3 × 10⁻⁴ s⁻¹). The fact that identical reactivation rates were seen whether inhibition was induced with 8-azido-ATP or 8-azido-ADP argues strongly that the inhibited Pgp species contains vanadate-trapped 8-azido-ATP at catalytic sites. This is consistent with previous work (Urbatsch et al., 1995), which established that vanadate-trapped ADP was the inhibitory species when Pgp was preincubated with ATP and vanadate, and with the fact that 8-azido-ATP is known to be a good substrate for hydrolysis by Pgp (Urbatsch et al., 1994). The reactivation of Pgp ATPase after inhibition with 8-azido-adenine nucleotide and vanadate was notably faster than was seen previously after inhibition with
adenine nucleotide and vanadate. Thus the fact that reactivation was slower in the case of Co-8-azido-nucleotide proved technically advantageous.

Permanent inactivation of Pgp ATPase was obtained in samples that had been inhibited by preincubation with vanadate and 200 μM Co-8-azido-ATP and then subjected to UV irradiation as described under "Experimental Procedures." Samples that had been so treated remained 91% inhibited after 4 h of incubation at 37 ºC. Thus, in contrast to the data in Fig. 1B, there was now essentially no reactivation, confirming that 8-azido-adenine nucleotide had been trapped in catalytic sites.

Dependence of Vanadate-induced Inhibition of Pgp ATPase Activity on Co-8-azido-ATP Concentration—The dependence of vanadate-induced inhibition of Pgp ATPase activity on the concentration of Co-8-azido-ATP was examined, and the concentration required for 50% inhibition was found to be 3 μM (Fig. 2). Maximal (95%) inhibition was reached at ~80 μM. We determined the amount of radioactively labeled nucleotide in the eluates obtained from centrifuge columns after preincubation of plasma membranes with vanadate and Co-8-azido-[α-32P]ATP (80 μM), so as to achieve 95% inhibition of Pgp ATPase, and the calculated stoichiometry of binding (see "Experimental Procedures") was 0.94 mol of nucleotide/mol of Pgp. Therefore, the vanadate trapping of Co-8-azido-adenine nucleotide at just one catalytic site per Pgp molecule brought about 100% inhibition of ATPase activity, consistent with what was seen earlier with Mg-adenine nucleotide (Urbatsch et al., 1995).

Photolabeling of the Two Pgp Nucleotide Sites by Vanadate-trapped 8-Azido-adenine Nucleotide—Pgp has two predicted nucleotide-binding sites, and it was therefore of interest to determine at which site vanadate trapping of nucleotide occurred when Pgp was maximally inhibited by a single mol of trapped nucleotide. We previously established (Urbatsch et al., 1995) that when CR1R12 plasma membranes were preincubated with vanadate and 200 μM Mg-8-azido-[α-32P]ATP to induce inhibition of Pgp ATPase, then the eluates from centrifuge columns were subjected to UV irradiation, the Pgp was specifically photolabeled. Whereas if vanadate was omitted, no photolabeling occurred. We first confirmed here that the same result was seen when 200 μM Co-8-azido-[α-32P]ATP was used instead of Mg-8-azido-[α-32P]ATP (data not shown).

We then performed experiments using a range of concentrations of Co-8-azido-[α-32P]ATP from 80 μM down to 5 μM. This concentration range produced inhibition of Pgp ATPase from 95% down to 50% (see Fig. 2), and therefore the stoichiometry of trapped nucleotide would range from ~1 mol/mol to around 0.6 mol/mol. Fig. 3 (lanes 1–4) shows the photolabeling of Pgp in plasma membranes after vanadate trapping with Co-8-azido-[α-32P]ATP at concentrations of 80, 20, 10, and 5 μM. Direct counting of the Pgp bands excised from SDS gels showed that 26–54% of the trapped 32P in the centrifuge column eluates became covalently attached to Pgp on UV irradiation and survived gel electrophoresis. For comparison, photoincorporation of vanadate-trapped azido-nucleotide analogs in myosin ranged from a few percent to 70% (Yount et al., 1992).

Georges et al. (1991) showed that Pgp in Chinese hamster ovary cell plasma membranes can be fragmented into N-terminal and C-terminal "halves" by mild trypsin digestion. Each half contains one of the predicted nucleotide-binding domains. The N-terminal (glycosylated) fragment has an apparent molecular size of 100 kDa and the C-terminal fragment of 65 kDa on SDS gels. We previously confirmed, using the C219 monoclonal antibody, that this technique is reproducible with CR1R12 cell plasma membranes (Al-Shawi et al., 1994). Fig. 3 (lanes 5–8) shows the results obtained when the photolabeled Pgp was digested with trypsin into the two halves before running on SDS gels. It is seen that even at the lowest concentration of nucleotide used, both halves of Pgp were labeled. The distribution of the radioactivity between the two halves as determined by direct counting of bands excised from gels was: N-terminal, 53%; C-terminal, 47%. This ratio was similar at all the nucleotide concentrations studied (the variation was ±9%). The fact that both nucleotide sites became labeled at all the nucleotide concentrations used showed that vanadate trapping of nucleotide in N- or C-terminal nucleotide sites occurred non-selectively.

Similar experiments to those shown in Fig. 3 were performed using Mg-8-azido-[α-32P]ATP, and they confirmed the above conclusions. Briefly, full inhibition of Pgp ATPase was obtained with ~1 mol of trapped Mg-nucleotide/mol of Pgp, and using a range of concentrations of Mg-nucleotide which yielded 36–74% inhibition of ATPase activity by vanadate trapping, it was found after subsequent UV irradiation and mild trypsin digestion of the samples that both halves of Pgp became photolabeled (data not shown).

Competition between Phosphate and Vanadate—Further studies on the mechanism of vanadate-induced inhibition of Pgp ATPase were made possible by investigating the effects of phosphate. Fig. 4A shows the effects of phosphate on vanadate-induced inhibition with 200 μM MgATP in plasma membranes. In the absence of phosphate (diamonds) the concentration of vanadate producing half-maximal inhibition was 4 μM, whereas the presence of 200 mM NaPi (open circles) increased this value to 23 μM. These data indicated that P_i competes with V_i, with K_i(P_i) = 38 mM. Fig. 4A (closed circles) shows data obtained in the presence of 133 mM NaSO_4, added to mimic the ionic strength provided by 200 mM NaPi. Here the concentration of vanadate producing half-maximal inhibition was 8 μM. Comparison of the "plus NaSO_4" data with the "plus NaPi," data yielded a K_i(P_i) of 100 mM.

Fig. 4B shows a parallel experiment using 200 μM MgADP. Here, the concentration of vanadate producing half-maximal inhibition was 9 μM (no P_i, squares) or 100 μM (plus 200 mM NaPi, open inverted triangles). The calculated K_i(P_i) from these data was 22 mM, and inhibition was again competitive. The closed inverted triangles show similar data obtained in the presence of 133 mM Na_2SO_4. Here the concentration of vanad-
date producing half-maximal inhibition was 26 \( \mu M \), corresponding to a \( K_i \) of 70 mM.

Further experiments were performed in which \( V_i \) concentration was held constant at 200 \( \mu M \), and MgATP or MgADP concentration was varied from zero to 200 \( \mu M \) in the presence or absence of 200 mM Pi. There was no significant effect of Pi on the observed inhibition at different nucleotide concentrations.

Effects of High Phosphate Concentrations on MgATP Hydrolysis—Fig. 5 shows one typical experiment performed to determine the effect of 200 mM NaPi on ATPase activity of Pgp in plasma membranes at varying concentrations of MgATP. In order to control for ionic strength, Na\( _2 \)SO\( _4 \) (133 mM) was included in the assays when Pi was not present. In separate experiments it was confirmed that Na\( _2 \)SO\( _4 \) at this concentration had no significant effect on \( V_{max} \) or \( K_m \) (MgATP) of Pgp in plasma membranes. The average data of three experiments showed that 200 mM Pi reduced \( V_{max} \) from 1.2 to 0.6 \( \mu mol \) of ATP hydrolyzed per min per mg of membrane protein, while \( K_m \) (MgATP) was reduced from 1.5 mM to 1.2 mM. These experiments showed that Pi is a weak, mixed-type inhibitor of ATP hydrolysis and that the concentration of Pi needed to reduce \( V_{max} \) by 50% is approximately 200 mM.

**DISCUSSION**

We used the technique of vanadate-induced inhibition by trapped nucleotide to study catalytic properties of P-glycoprotein in plasma membranes from multidrug-resistant CR1R12 Chinese hamster ovary cells. It was demonstrated that in presence of vanadate plus 8-azido-[\( \alpha \)-\( ^32P \)]ATP and either Mg\( ^{2+} \) or Co\( ^{2+} \), 8-azido-adenine nucleotide became stably trapped in Pgp catalytic sites. Trapping of 1 mol of nucleotide per mol of Pgp caused complete inhibition of ATPase. The evidence presented here and previously (Urbatsch et al., 1995) indicates that nucleoside diphosphate is the trapped inhibitory species. UV irradiation of inhibited Pgp caused permanent inactivation and revealed that both halves of Pgp became photolabeled. Vanadate trapping of nucleotide in the N- or C-terminal nucleotide sites appeared to occur non-selectively. The true distribution of vanadate-trapped nucleotide in the two sites might be obscured by differential covalent attachment upon UV irradiation or loss of covalent label on SDS-gel electrophoresis. Nevertheless, the presence of radioactive label in both halves of Pgp provides convincing evidence that both nucleotide sites in intact Pgp are capable of hydrolyzing 8-azido-ATP to 8-azido-ADP and thus forming the stably inhibited Pgp-8-azido-ADP-V\( i \) complex. Furthermore, vanadate trapping of nucleotide at either of the

**Fig. 3. Photolabeling of Pgp in plasma membranes after vanadate trapping with Co-8-azido-[\( \alpha \)-\( ^32P \)]ATP.** Vanadate-induced inhibition in the presence of varied concentrations of 8-azido-[\( \alpha \)-\( ^32P \)]ATP, 200 \( \mu M \) vanadate, and 3 mM Co\( \text{SO}_4 \) and subsequent UV irradiation of inhibited samples were performed as described under "Experimental Procedures." The photolabeled samples were run on SDS gels and subjected to autoradiography. Lanes 1–4, 80, 20, 10, and 5 \( \mu M \) nucleotide; lanes 5–8, same as lanes 1–4 except that the samples were subjected to mild trypsin digestion to fragment the Pgp into N- and C-terminal halves before being applied to SDS gels. The position of N- and C-terminal halves of Pgp on SDS gels was confirmed using immunoblotting with C219 anti-Pgp monoclonal antibody.

**Fig. 4. Competition between phosphate and vanadate during vanadate-induced inhibition of Pgp ATPase activity in plasma membranes.** Vanadate-induced inhibition of Pgp ATPase activity was carried out as described under "Experimental Procedures" using varying concentrations of vanadate. Panel A, using 200 \( \mu M \) MgATP: diamonds, no addition; open circles, with 200 mM sodium phosphate; closed circles, with 133 mM Na\( _2 \)SO\( _4 \). Panel B, using 200 \( \mu M \) MgADP: squares, no addition; open inverted triangles, with 200 mM sodium phosphate; closed inverted triangles, with 133 mM Na\( _2 \)SO\( _4 \).
two sites must be able to completely prevent hydrolysis at both sites in order to give complete inhibition of ATPase activity. Therefore, both nucleotide sites in Pgp are potentially catalytically active, but they are not independent; indeed there is very strong cooperative interaction between them. One likely possibility is that the two sites alternate in catalysis.

The data presented here are consistent with previous results (Al-Shawi et al., 1994), which indicated that ATP and $\delta$-azido-ATP bind to both of the two nucleotide-binding sites. Much higher concentrations of $\delta$-azido-ATP were used previously than here because vanadate was not used, and this explains why labeling stoichiometries of up to 2 mol/mol of Pgp were seen in the earlier work. It was also seen previously that NBD-Cl gave full inhibition of ATPase at a labeling stoichiometry of 1 mol/mol of Pgp. This also is consistent with the results obtained here. Interestingly, NBD-Cl reacted predominantly in the C-terminal half, this preference being presumably due to a specific reactive residue in the C-terminal nucleotide site, which is either absent or less reactive in the N-terminal nucleotide site. N-Ethylmaleimide, in contrast, appeared to react in both nucleotide sites.

The data presented here are also consistent with previous mutagenic analyses of Pgp, which showed that introduction of point mutations into either or both nucleotide sites rendered the Pgp completely inactive (Azzaria et al., 1989; Roninson, 1992), and also with the data of Loo and Clarke (1994), which showed that either nucleotide site is potentially capable of ATP hydrolysis. It seems likely that ATPase activity in the experiments of Loo and Clarke (1994) resulted from formation of homodimers of the expressed "half-molecules."

In Scheme 1 we propose a minimal reaction pathway for vanadate-induced inhibition of Pgp. This scheme draws on the models of Loo and Clarke (1994) which is either absent or less reactive in the N-terminal nucleotide site. N-Ethylmaleimide, in contrast, appeared to react in both nucleotide sites.

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