Inhibition of P-Glycoprotein ATPase Activity by Beryllium Fluoride†

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ABSTRACT: ATPase activity of P-glycoprotein (multidrug-resistance protein) was found to be potently inhibited by beryllium fluoride (BeF_x) in combination with MgATP, MgADP, or corresponding Mg-8-azido-nucleotides. Inhibition was due to trapping of nucleoside diphosphate at catalytic sites. Full inhibition was achieved on trapping of 1 mol of nucleotide per mol of Pgp. Reactivation was slow ($t_{1/2}$ = 32 min at 37 °C), and release of trapped nucleotide correlated with recovery of ATPase. Trapping of 8-azido-ADP followed by UV irradiation yielded permanent inactivation and specific labeling of Pgp in plasma membranes. Both N- and C-terminal nucleotide binding sites were labeled. These findings give strong confirmation of the concepts that in intact Pgp both nucleotide sites are active in MgATP hydrolysis, and that they interact strongly. The characteristics of inhibition by BeF_x were similar in general to those seen with vanadate. However, PP_i gave strong protection against BeF_x inhibition, and in this respect, inhibition by BeF_x was clearly different from vanadate inhibition.

P-glycoprotein (Pgp), also called the multidrug-resistance protein, is a plasma-membrane-located glycoprotein. *In vitro* it has been shown to confer multidrug-resistance phenotype on cells. The mechanism by which this resistance is achieved is not yet understood in detail, but it is clear that Pgp can exclude drugs from cells in an ATP-dependent manner. The most commonly considered current hypothesis is that Pgp acts as an ATP-driven drug-export pump. Multidrug-resistance is an important obstacle in treatment of human cancer, and for this reason there is considerable interest in the potential role of Pgp in rendering tumor cells resistant to chemotherapeutic drugs (Endicott & Ling, 1989; Gottesman & Pastan, 1993; Gros & Buschman, 1993; Shapiro & Ling, 1995; Gottesman, 1993; Germann, 1996).

Pgp molecules from human and rodents show similar amino acid sequences and are about 1280 residues in length, with two nucleotide-binding sites (NBS). It has been established that Pgp shows substantial ATPase activity in the absence of added drugs (called "basal activity") and that the ATPase is stimulated several-fold by drugs [reviewed in Senior et al. (1995a)]. The properties of the catalytic sites have been characterized extensively by kinetic methods (Senior et al., 1995b; Sharom et al., 1995) and are known to be of low affinity for MgATP ($K_M \sim 1$ mM) and of low selectivity for nucleotides; however, such approaches have not yet shed light on reasons for the presence of two NBS per Pgp molecule. Covalent labeling studies with NBD-Cl, 8-azido-ATP, and NEM (Georges et al., 1991; Al-Shawi et al., 1994; Loo & Clarke, 1995a) have indicated that both NBS bind MgATP, and that blocking either site with reagent fully prevents ATP hydrolysis. Mutations introduced independently into the "P-loop" sequence of either NBS1 or NBS2 were found to prevent conferral of the multidrug-resistance phenotype in cells and to fully prevent ATP hydrolysis (Azzaria et al., 1989; Loo & Clarke, 1995b; Muller et al., 1996). These data established that both NBS must be intact for normal function and raised the possibility that interaction between them is required as part of the normal catalytic activity. Further experiments using the inhibitor vanadate (V_i) supported the latter concept (Urbatsch et al., 1995a,b) and indicated that both NBS have the ability to hydrolyze MgATP.

Beryllium fluoride (BeF_x)[1] is a potent inhibitor of ATPase enzymes; for example, it has been shown to inhibit F_1-ATPase (Issartel et al., 1991) and myosin (Phan & Reisler, 1992; Werber et al., 1992; Phan et al., 1993). In these cases inhibition results from the stable trapping of MgADP·BeF_x complex in the catalytic sites, and it is evident that BeF_x represents a valuable tool for investigations of the catalytic mechanism of ATPase enzymes. BeF_x also proved important for structural studies, and X-ray structure resolution of the myosin·MgADP·BeF_x complex (Fisher et al., 1995) demonstrated that this species has the conformation of the prehydrolysis, MgATP-bound complex. A further advantage of BeF_x is that it is optically silent, and therefore potentially useful for studies of conformational changes using reporter probes. Our laboratory is pursuing functional and structural studies of Pgp, and in this paper we report a detailed investigation of the inhibition of Pgp ATPase by BeF_x.

EXPERIMENTAL PROCEDURES

Preparation of Plasma Membranes. Plasma membranes were prepared from the multidrug-resistant Chinese hamster ovary cell line CR1R12 (Al-Shawi & Senior, 1993) as described previously (Urbatsch et al., 1995a). The membranes contained 15−20% (w/w) Pgp as a fraction of total membrane protein.

Assay of Pgp-ATPase Activity. ATPase activity was assayed using an ATP-regenerating system as described previously (Urbatsch et al., 1995a). Membrane protein, 10 μg or less, was added to 1 mL of assay medium at 37 °C

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[1] BeF_x is used as the abbreviation for beryllium fluoride complex because the exact composition is unknown. On the basis of NMR evidence it appears to be a mixture of hydroxyfluoroberyllates (Henry et al., 1993; Maruta et al., 1993).

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containing 10 mM ATP, 12 mM $MgSO_4$, 3 mM phosphoenolpyruvate, 0.33 mM NADH, 10 units of lactate dehydrogenase, 10 units of pyruvate kinase, and 40 mM Tris-HCl, pH 7.4. ATP hydrolysis, recorded as absorbance decrease at 340 nm, was linear in the range 0.33−0.01 mM NADH. Unless otherwise stated the ATPase activity was calculated from the absorbance decrease during the first 5 min, which was linear in all cases. EGTA (0.1 mM) and ouabain (2 mM) were included to eliminate Ca-ATPase and Na,K-ATPase activity, respectively, and 10 μM verapamil was included to maximally stimulate the Pgp. The Pgp-ATPase activity was around 1.3 μmol of ATP hydrolyzed/min/mg of membrane protein. Under the assay conditions described, the plasma membranes (which are prepared in 0.25 M sucrose-containing buffer) are osmotically-disrupted (Al-Shawi & Senior, 1993).

BeF_x-Induced Inhibition of Pgp. Plasma membranes (10 μg) were incubated with 200 μM $BeSO_4$, 1 mM NaF, 200 μM nucleotide, 2 mM $MgSO_4$, 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 the conditions are detailed in tables and 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 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. Unless otherwise stated the centrifuge column step was carried out at 23 °C. Control experiments using [α-^{32}P]ATP showed that <0.002% of the applied nucleotide eluted from the columns in the absence of membranes.

Characterization of Effects of PP_i and *P_i on BeF_x-Induced Inhibition of Pgp*. BeF_x inhibition of Pgp ATPase in plasma membranes was induced as above, except that MgATP concentration was 200 μM, NaF was 1 mM, and BeF_x concentration was varied from 0.1 μM to 1 mM. In addition, $NaPP_i$ was added at 50 μM, 100 μM, or 1 mM final concentration or NaP_i was added at 2, 5, 7.5, 20, 50, or 200 mM. Degree of inhibition was assayed as above, and curves of inhibition vs BeF_x concentration were derived. Apparent $K_i(PP_i)$ was calculated from

$$K_i(PP_i) = (EC_{50}.[PP_i])/(EC_{50app} - EC_{50})$$

where EC_{50} is the concentration of BeF_x for 50% inhibition in absence of PP_i (23 μM, see Results), $[PP_i]$ is the concentration of PP_i added, and EC_{50app} is the apparent EC_{50} in presence of $[PP_i]$. Apparent $K_i(P_i)$ was calculated in an analogous fashion.

Determination of Pgp Content of Plasma Membranes and Stoichiometry of Trapped Radioactive Nucleotide. The stoichiometry of BeF_x-trapped nucleotide in Pgp was calculated from the amount of radioactive nucleotide in the centrifuge column eluates after BeF_x-induced inhibition and the amount of Pgp in the plasma membranes in the same eluates. Control experiments using plasma membranes from the AUXB1 parent cell line, containing negligible Pgp, showed a small amount of radioactivity trapped in the presence of BeF_x and MgATP, which was subtracted from the data for CR1R12 cell plasma membranes. The amount of Pgp in the membranes was analyzed as described by Al-Shawi et al. (1994). Briefly, the centrifuge column eluates were run on SDS gels, stained with Coomassie Blue, and subjected to laser densitometry using a Molecular Dynamics Personal Densitometer equipped with MD Image Quant software, version 3.3. We have established previously that this method is reliable as a means of determining the Pgp concentration in membrane samples (Al-Shawi et al., 1994). The molecular size of Pgp was taken as 141 kDa.

HPLC Analysis of BeF_x-Trapped Nucleotides. The centrifuge column eluates were collected directly into 100 μL of 10% (w/v) ice-cold trichloroacetic acid and placed on ice. The samples were centrifuged to precipitate the protein, 150 μL of the supernatant was added to 1.85 mL of 10 mM H_3PO_4 solution, and the whole sample was applied to a 150 × 10 mm Fractogel EMD TMAE-650S column, 25−40 μM particle size (EM Science). The column was eluted at a flow rate of 0.5 mL/min, using a linear gradient of 10 mM H_3PO_4 plus 0−100% 750 mM KH_2PO_4, pH 4.4 (0−60 min), followed by 750 mM KH_2PO_4, pH 4.4 (20 min). Fractions of 0.5 mL were collected, and ^{32}P was determined by Cerenkov counting. The elution times of authentic ADP and ATP were 46 and 63 min, respectively.

Routine Procedures. SDS gel electrophoresis, immunoblotting, and protein assay by bicinchoninic acid method in presence of 1% SDS were all performed as previously described (Al-Shawi & Senior, 1993).

Materials. [α-^{32}P]ATP was from Amersham, and [α-^{32}P]-8-azido-ATP was from Research Products International. Tissue culture materials were from BRL Life Technology Inc. Lactate dehydrogenase (cat. no. 127868) and pyruvate kinase (cat. no. 109053), both supplied in 50% glycerol, were from Boehringer Mannheim. C219 anti-Pgp monoclonal antibody was from Signet Laboratories.

RESULTS

Long-Lived Inhibition of P-Glycoprotein ATPase Activity by Beryllium Fluoride and Conditions for Reactivation. The experiments to be described were performed using purified plasma membranes from the CR1R12 Chinese hamster ovary cell line (Al-Shawi & Senior, 1993). The plasma membranes contained 15−20% (w/w) Pgp as a fraction of total membrane protein and the specific activity of the Pgp ATPase in presence of verapamil was around 1.3 units/mg of membrane protein, assayed as described in Experimental Procedures. Ouabain and EGTA were included in assays to eliminate Na,K-ATPase and (very low) Ca-ATPase, respectively, and mitochondrial and ecto-ATPase activities were absent. We have characterized this preparation extensively in past studies (Al-Shawi & Senior, 1993; Al-Shawi et al., 1994; Urbatsch et al., 1995a,b).

We found that preincubation of plasma membranes with MgATP, $BeSO_4$, and NaF, followed by passage of the membranes through a centrifuge column to remove unbound ligands, resulted in strong inhibition of Pgp ATPase activity. When the membranes from the centrifuge column eluate were further incubated in buffer (40 mM Tris-HCl, pH 7.4, 0.1 mM EGTA), the Pgp slowly reactivated in a temperature-dependent manner, and full initial activity was eventually recovered. Figure 1 shows a typical experiment. The $t_{1/2}$ values for reactivation were 37 °C, 32 min; 30 °C, 83 min; 23 °C, >3 h; 4 °C, >12 h. At 37 °C the rate of reactivation was the same whether MgATP (10 mM) and/or verapamil (10 μM) were present or not. If MgATP, $BeSO_4$, or NaF was omitted from the preincubation, there was little inhibition

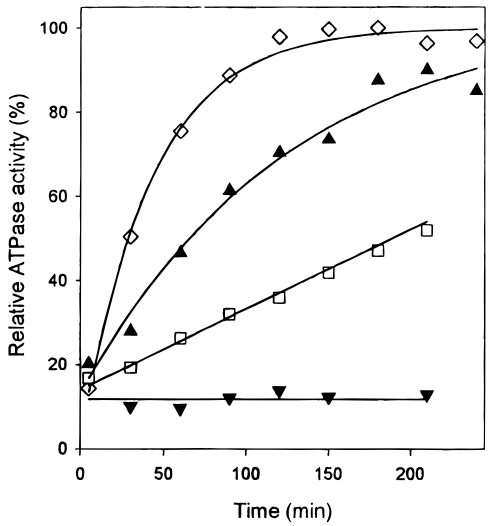

FIGURE 1: Inhibition of P-glycoprotein ATPase activity by BeF_x and dependence of reactivation on temperature. Plasma membranes from CR1R12 cells (10 μg) were preincubated at 37 °C for 20 min in 100 μL final volume containing 200 μM $BeSO_4$, 1 mM NaF, 200 μM MgATP, 2 mM $MgSO_4$, 0.1 mM EGTA, and 40 mM Tris-HCl, pH 7.4. The whole sample was passed through a centrifuge column at 23 °C, previously equilibrated in 40 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, to remove unbound ligands. Eluates were incubated at various temperatures, and the recovery of ATPase activity was followed (see Experimental Procedures for further details): (◇) 37 °C; (▲) 30 °C; (□) 23 °C; (▼) 4 °C (in this case the centrifuge column elution was also performed at 4 °C).

Table 1: Inhibition of P-Glycoprotein ATPase Activity by BeF_x[a]

nucleotide	Mg^{2+}	$BeSO_4$	NaF	other additions	ATPase inhibition (%)
−	+	+	+		≤5
ATP	+	+	+		90[b]
ATP	+	−	+		≤4
ATP	+	+	−		≤8
ATP	−	+	+	2 mM EDTA	0
ATP	+	+	+	1 mM PP_i	0
ATP	+	+	+	20 mM P_i	67
ATP	+	+	+	50 mM P_i	25
ATP	+	+	+	33 mM Na_2SO_4	90
ADP	+	+	+		90[b]
ADP	−	+	+	2 mM EDTA	0
ADP	+	+	+	1 mM PP_i	0
ADP	+	+	+	50 mM P_i	25
−	+	+	+	1 mM PP_i	0
−	+	+	+	50 mM P_i	0

[a] Conditions are described in detail in Experimental Procedures. Briefly, plasma membranes (10 μg/100 mL) were incubated with nucleotide (1 mM), $MgSO_4$ (3 mM), $BeSO_4$ (0.2 mM), NaF (1 mM), and other additions as indicated and then passed through centrifuge columns, and ATPase activity was assayed. [b] Range was 85−95% in a large number of experiments.

of the Pgp ATPase activity (Table 1, lines 1−5). Thus it was apparent that BeF_x in conjunction with nucleotide led to long-lived inhibition of Pgp.

PP_i and P_i were found to be effective in preventing inhibition (Table 1, lines 6−8); control experiments showed that this was not due to increased ionic strength (Table 1, line 9) and that neither PP_i nor P_i caused inhibition if substituted for nucleotide (Table 1, lines 14 and 15).

MgADP could substitute for MgATP to induce inhibition (Table 1, line 10). It was found that the rate of reactivation of Pgp ATPase activity was exactly the same whether MgATP or MgADP was present in the preincubation with

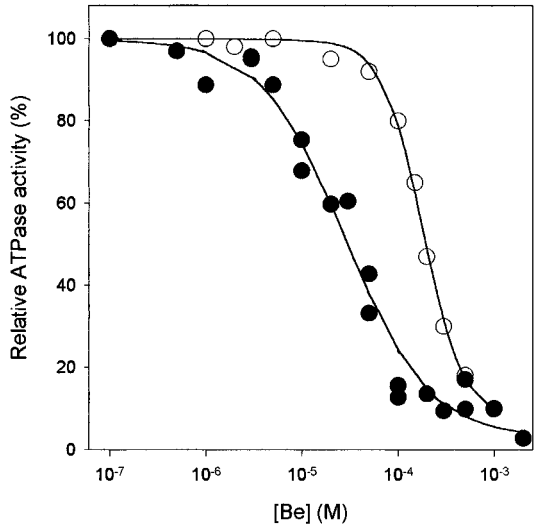

FIGURE 2: Dependence of inhibition of Pgp on the concentration of BeF_x. Inhibition of Pgp ATPase in plasma membranes was carried out as in Experimental Procedures. NaF concentration was 1 mM, MgATP concentration was 0.2 mM, and $BeSO_4$ concentration was varied; (●) no further additions; (○) 0.1 mM $NaPP_i$ added.

BeF_x, suggesting that BeF_x inhibits by trapping MgADP in the catalytic sites. PP_i and P_i also protected with MgADP (Table 1, lines 12 and 13).

Divalent cation was required for inhibition, as inclusion of EDTA in the preincubation demonstrated (Table 1, lines 5 and 11). With both ATP or ADP, Mn, and Co were effective substitutes for Mg, yielding the same degree of inhibition. When inhibition was achieved using Mn or Co with ATP, the $t_{1/2}$ values for reactivation at 37 °C were Mn, 47 min, and Co, 25 min; however, the samples with CoATP reactivated to only 30% of the original (data not shown).

Dependence of Inhibition on Concentration of BeF_x, MgATP, or MgADP. Figure 2 (closed circles) shows the dependence of inhibition of Pgp ATPase on the $BeSO_4$ concentration at a constant NaF concentration of 1 mM in presence of MgATP. Half-maximal inhibition was achieved at a concentration of 23 μM $BeSO_4$ with MgATP (18 μM with MgADP). Figure 3A and 3B show the dependence of inhibition on MgATP or MgADP concentrations at constant 0.2 mM $BeSO_4$/1 mM NaF. Concentrations of MgATP or MgADP required for half-maximal inhibition were 16 and 10 μM, respectively. Considering that for ATPase activity the K_M(MgATP) is ∼1 mM and the K_i(MgADP) is ∼0.5 mM (Senior et al., 1995b), it is evident that BeF_x greatly increases the apparent affinity of the catalytic sites for both nucleotides.

Time Course of Inhibition with MgATP and MgADP. Figure 4 shows the time course of inhibition with MgADP or MgATP in the presence of BeF_x. Whereas inhibition occurred rapidly with MgATP, with MgADP the time course was slower than expected for a simple binding reaction, suggesting that a slower reaction, probably a protein conformational change, was rate limiting. A similar mechanism was proposed for inhibition of myosin by BeF_x with MgADP (Phan & Reisler, 1992).

Demonstration that BeF_x Inhibits P-Glycoprotein ATPase Activity by Trapping Nucleotide in the Catalytic Site and that Reactivation Correlates with Release of Trapped Nucleotide. Pgp was inhibited by preincubation of plasma membranes with BeF_x and [α-^{32}P]MgATP, then passed

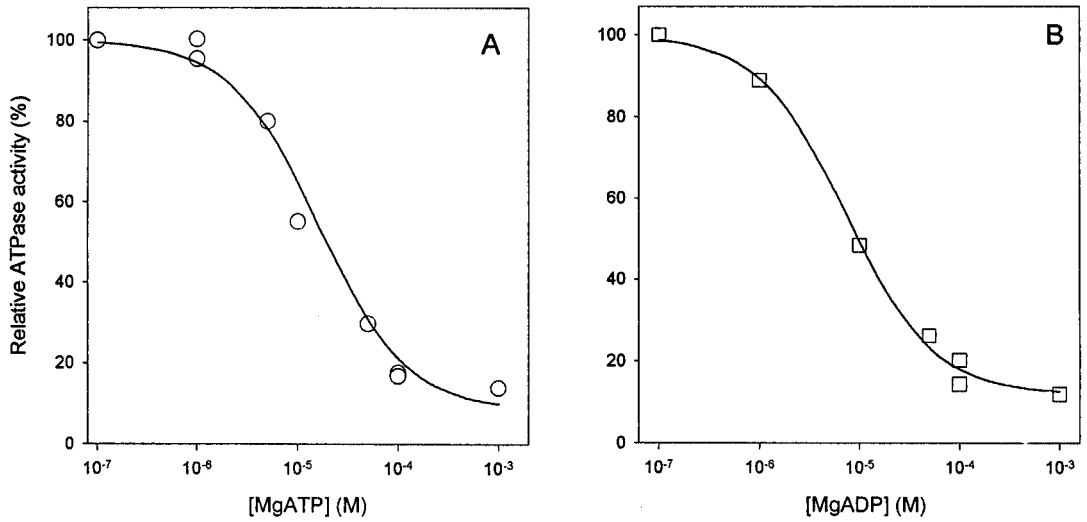

FIGURE 3: Dependence of inhibition of Pgp on the concentration of MgATP and MgADP. Inhibition of Pgp ATPase in plasma membranes was carried out as in Experimental Procedures. NaF concentration was 1 mM, $BeSO_4$ concentration was 0.2 mM, and either the MgATP concentration (A) or the MgADP concentration (B) was varied.

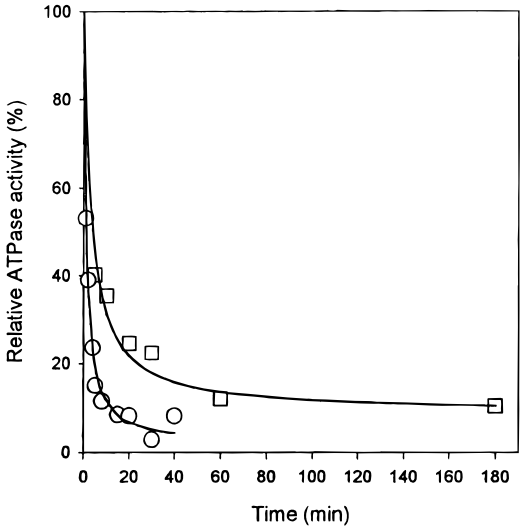

FIGURE 4: Time course of onset of inhibition of Pgp by BeF_x in the presence of MgATP or MgADP. Plasma membranes were incubated with $BeSO_4$ (0.2 mM), NaF (1 mM), and nucleotide (0.2 mM) as described in Experimental Procedures, for various times, and were then passed through centrifuge columns and assayed for ATPase activity: (○) MgATP; (□) MgADP.

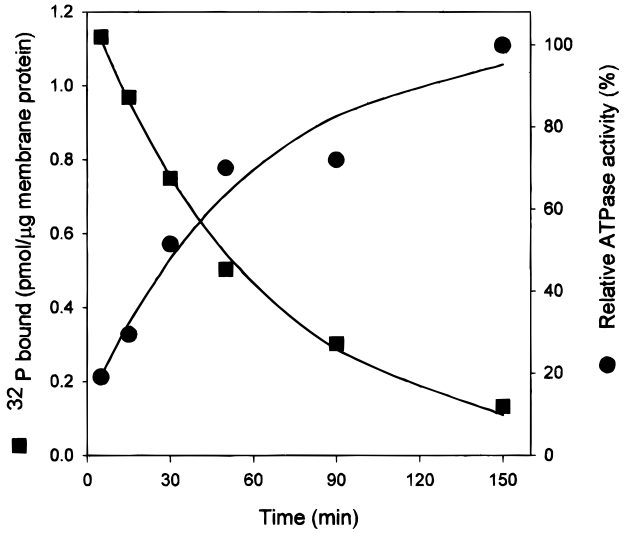

FIGURE 5: Demonstration that release of trapped radioactive nucleotide correlates with reactivation of ATPase activity. Plasma membranes from CR1R12 cells (Pgp content = 15% of total membrane protein) were preincubated with $BeSO_4$ (0.2 mM), NaF (1 mM), and [α-^{32}P]ATP (0.2 mM) as described under Experimental Procedures, except that 20 mM Na_2SO_4 was included to reduce nonspecific binding of radioactive nucleotide. The membranes were passed through centrifuge columns to remove unbound ligands (time zero), and the eluates were incubated at 35 °C. At various time intervals, aliquots were passed through second centrifuge columns and ATPase activity and bound [α-^{32}P]nucleotide were assayed. The lines are nonlinear regression fits to the data. Amounts of bound radioactivity were corrected for small amounts of bound radioactivity seen in parent AUXB1 membranes (which contain negligible amounts of Pgp) treated in the same way.

through a centrifuge column to remove unbound ligands. The plasma membranes in the eluates contained bound radioactive nucleotide equal in amount to 0.88 and 1.06 mol/mol of Pgp (average = 0.97) in two experiments. This indicated that trapping of nucleotide by BeF_x in a single Pgp catalytic site was sufficient to fully exhibit ATPase activity. As is shown in Figure 5, when the inhibited membranes were incubated so as to reactivate the ATPase, release of bound nucleotide occurred coincident with reactivation. The rate of reactivation of the Pgp ATPase and rate of release of radioactive nucleotide were essentially the same ($t_{1/2}$ for reactivation = 41 min, $t_{1/2}$ for radioactive nucleotide release = 43 min, temperature was 35 °C in Figure 5).

Demonstration that the Nucleotide Trapped by BeF_x Is MgADP. Plasma membranes were preincubated with BeF_x and [α-^{32}P]MgATP and then passed through centrifuge columns, and the eluates were collected directly into an equal volume of ice-cold 10% (w/v) trichloroacetic acid. The radioactive nucleotide in the eluate was then analyzed by HPLC. As can be seen in Figure 6A, a single major radioactive peak was seen, corresponding to ADP. There was no ATP in the eluate from the centrifuge column. For comparison, Figure 6B shows a similar experiment in which vanadate was used instead of BeF_x, and as expected from previous work (Urbatsch et al., 1995a) ADP was the single major radioactive peak in this experiment. These data establish therefore that BeF_x inhibits Pgp ATPase by trapping MgADP in a catalytic site.

Use of 8-Azido-ATP to Further Investigate Inhibition of P-Glycoprotein by BeF_x. The photoaffinity label 8-azido-

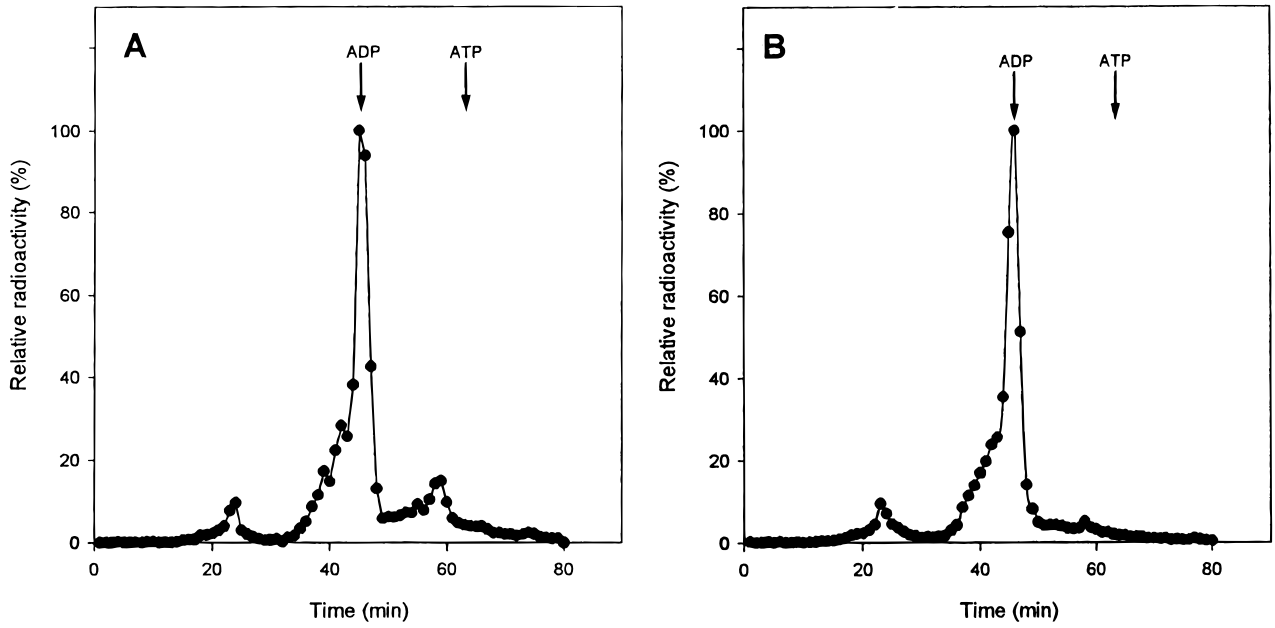

FIGURE 6: HPLC analysis of the BeF_x-trapped nucleotide. (A) Plasma membranes were preincubated with $BeSO_4$ (0.2 mM), NaF (1 mM), and [α-^{32}P]ATP (0.2 mM). Trapped radioactive nucleotide was analyzed by HPLC as described under Experimental Procedures. 100% is defined arbitrarily as the radioactivity present in the highest ADP-containing fraction. (B) Vanadate (200 μM) was substituted for BeF_x, and [α-^{32}P]ATP concentration was 50 μM. Other conditions were as in A. Inhibition of Pgp ATPase ≥85% occurred under these conditions.

ATP is a good hydrolysis substrate of Pgp (Al-Shawi et al., 1994). Here we found that when plasma membranes were preincubated with BeF_x and Mg-8-azido-ATP and then passed through a centrifuge column to remove unbound ligands, inhibition of the Pgp ATPase activity occurred (80−90%), just as with MgATP. Half-maximal inhibition occurred at 4.1 μM Mg-8-azido-ATP concentration in presence of 0.2 mM $BeSO_4$/1 mM NaF, and reactivation of ATPase occurred with $t_{1/2}$ = 10.5 min at 37 °C. Mg-8-azido-ADP plus BeF_x also induced inhibition, the $t_{1/2}$ of reactivation being 11 min. With Co-8-azido-ATP and Co-8-azido-ADP inhibition was also seen, and the measured $t_{1/2}$ values for reactivation of ATPase at 37 °C were Co-8-azido-ATP, 12.6 min, and Co-8-azido-ADP, 12.9 min. Thus, 8-azido-nucleoside diphosphate was trapped at the catalytic site by BeF_x.

Photolabeling of Pgp in plasma membranes was achieved using BeF_x-trapped 8-azido-ADP. After preincubation of plasma membranes with BeF_x and Mg-8-azido-[α-^{32}P]ATP, followed by elution through a centrifuge column, the eluates were subjected to UV irradiation. This produced permanent inactivation of Pgp ATPase, that is to say, no recovery of ATPase activity was seen on incubation at 37 °C for up to 4 h. The UV-irradiated membranes were subjected to SDS gel electrophoresis and autoradiography (Figure 7), and the results showed that the Pgp in the membranes became labeled specifically. (The faint band indicated by the lower arrow is a proteolysis product of Pgp, as was shown by immunoblotting with C219 monoclonal antibody.)

It was documented previously (Georges et al., 1991) that mild trypsin digestion cleaves Pgp in plasma membranes into an N-terminal "half", which is glycosylated, has apparent mass of ∼100 kDa on SDS gels, and contains NBS1, and a C-terminal "half", with apparent mass of 65 kDA, containing NBS2. Here, plasma membranes were preincubated with BeF_x and Mg-8-azido-[^{32}P]ATP, passed through centrifuge columns to remove unbound ligand, and subjected to UV irradiation. Conditions were chosen to achieve 50−100% inhibition by varying 8-azido-ATP concentration. Samples

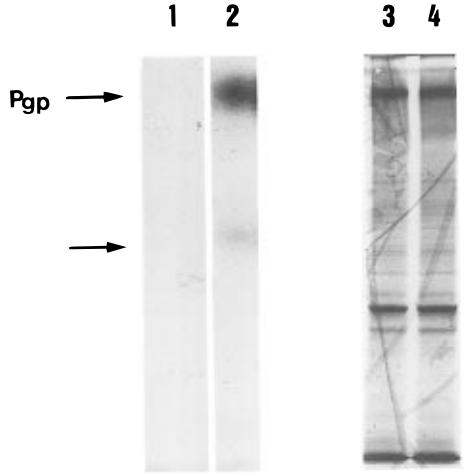

FIGURE 7: BeF_x trapping of 8-azido-[α-^{32}P]nucleotide and subsequent photolabeling of Pgp. Plasma membranes were preincubated with 0.1 mM 8-azido-[α-^{32}P]ATP, 3 mM $MgSO_4$, and 1 mM NaF in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 0.2 mM $BeSO_4$. Unbound ligands were removed by passage through centrifuge columns. The eluates were placed on ice and irradiated for 2 min (λ = 254 nm, 1.1 mW/cm^2). Samples (20 μg) were subjected to SDS gel electrophoresis and then autoradiography (lanes 1 and 2) or staining with Coomassie Blue (lanes 3 and 4). Arrows indicate the position of Pgp (upper) and a proteolysis product (lower) identified by immunoblotting with C219 monoclonal antibody. When NaF was omitted but 0.2 mM $BeSO_4$ was included, the results were as in lanes 1 and 3.

were then subjected to mild trypsin digestion before they were run on SDS gels. The results of an autoradiogram (Figure 8) showed that both NBS became labeled by trapped 8-azido-ADP, with the C-terminal half apparently labeled to greater extent.

In order to further assess the distribution of label between NBS1 and NBS2, bands corresponding to the N- and C-terminal halves were excised from stained SDS gels and counted. It was found that the radioactivity was distributed between them as follows: N-terminal half, 34%

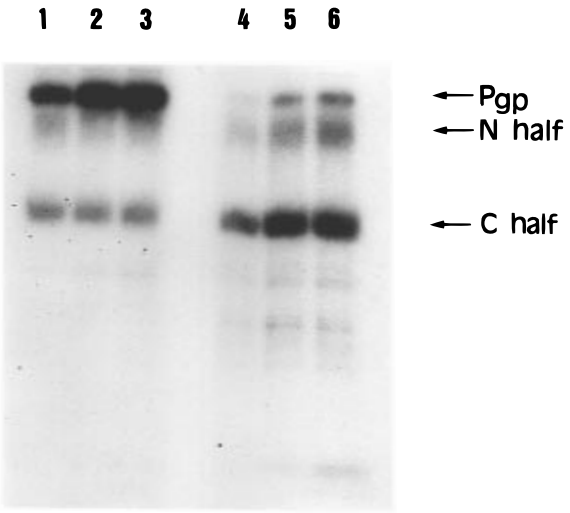

FIGURE 8: Photolabeling of Pgp in plasma membranes after BeF_x trapping with 8-azido-[α-^{32}P]ATP: effects of mild trypsin digestion. BeF_x-induced inhibition of Pgp in plasma membranes was carried out as described in Experimental Procedures, in presence of various concentrations of 8-azido-[α-^{32}P]ATP. The samples were passed through centrifuge columns and subjected to UV irradiation as in the legend to Figure 7. Photolabeled samples (20 μg) were run on SDS gels and subjected to autoradiography. Lanes 1−3: 4, 20, and 100 μM nucleotide, respectively. Lanes 4−6: same as lanes 1−3, except the samples were subjected to mild trypsin hydrolysis (0.05 μg of trypsin per 20 μg of sample, 37 °C for 10 min) to fragment the Pgp into N- and C-terminal halves, before being applied to the SDS gels. The positions of the N- and C-terminal halves (arrows) were confirmed by immunoblotting with C219 anti-Pgp monoclonal antibody.

(average of six different experiments, range = 27−41%); C-terminal half, 66% (range = 59−73%). It may be noted that the N-terminal half, which carries all of the Pgp glycosylation, runs diffusely on gels and thus shows lower-than-expected density in autoradiograms. In any case, we would emphasize that photolabeling experiments do not give a truly quantitative measure of nucleotide binding affinity or catalytic site occupancy because they are open to artifacts such as differential degree of covalent labeling of the N- and C-terminal halves and differential loss of label during subsequent manipulation of the protein. The data do clearly establish, however, that in intact Pgp in membranes both nucleotide sites bind and hydrolyze nucleoside triphosphate.

PP_i Competes with BeF_x and Protects Against Inhibition of P-Glycoprotein ATPase Activity. Table 1 had shown that when PP_i (1 mM) was included in the preincubation of plasma membranes with BeF_x and MgATP, inhibition of Pgp ATPase activity was completely prevented. This was further investigated by measuring the degree of inhibition obtained when BeF_x concentration was varied at fixed concentration of MgATP (0.2 mM) and in the presence of different concentrations of PP_i, as described in detail in Experimental Procedures. Degree of inhibition was found to be dependent on PP_i concentration, and the results indicated that there was competition between PP_i and BeF_x. Figure 2 (open circles) shows a typical experiment in which 0.1 mM PP_i was included. The calculated apparent $K_i(PP_i)$ was 18 μM (average of 10 experiments). In separate experiments BeF_x concentration was held constant while MgATP or MgADP concentration was varied in presence of different concentrations of PP_i. There was, however, no evidence of competition between PP_i and nucleotide. We tested whether PP_i affected ATPase activity, and we found that neither V_{max} nor K_M(MgATP) was changed by the inclusion of 1.0 mM $NaPP_i$ in the ATPase assay.

Analogous experiments were carried with P_i, and it was found that P_i also competed with BeF_x (apparent $K_i(P_i)$ = 3.2 mM, average of nine experiments) and that P_i did not compete with MgADP or MgATP.

Inhibition of ATPase Activity of Pure, Reconstituted Pgp by BeF_x. Pgp was purified and reconstituted in proteoliposomes as described (Urbatsch et al., 1994). Samples (90 μg of protein/mL) were preincubated with $BeSO_4$, NaF, and MgATP under the same conditions as used for plasma membranes in Experimental Procedures, and then 8 μL was added directly to 1 mL of ATPase assay buffer (which contained 0.1 mM EGTA and 50 μM verapamil). It was seen that the ATPase activity was initially inhibited by 80−90% in several experiments and that reactivation to full initial activity (4.9 μmol/min/mg of Pgp) occurred at 37 °C with $t_{1/2}$ = 39 min. When MgADP was substituted for MgATP in preincubation, similar results were observed. Therefore pure Pgp behaved similarly to plasma membrane Pgp with respect to BeF_x inhibition.

DISCUSSION

We have documented here that P-glycoprotein (Pgp) in plasma membranes is strongly inhibited by beryllium fluoride (BeF_x) in combination with Mg-nucleotide. MgATP, MgADP, Mg-8-azido-ATP, and Mg-8-azido-ADP were all effective. In each case inhibition was long-lived and involved trapping of Mg-nucleoside-diphosphate at a catalytic site. Reactivation occurred slowly, in a temperature-dependent fashion, and paralleled release of trapped nucleotide. Full ATPase activity was ultimately recovered. BeF_x was seen to markedly increase the apparent affinity of the catalytic site for all of the nucleotides. Trapped Mg-8-azido-ADP specifically photolabeled Pgp in plasma membranes upon UV irradiation and permanently inactivated Pgp ATPase.

Full inhibition of Pgp-ATPase activity correlated with trapping of 1 mol of MgADP per mol of Pgp, therefore when one of the NBS contained trapped nucleotide, neither site was active. This provides evidence that the two nucleotide sites in Pgp interact together in catalysis. Photolabeling experiments with 8-azido-Mg-ATP revealed that both nucleotide sites became labeled, demonstrating that both NBS are capable of nucleoside triphosphate hydrolysis, and that trapping of nucleotide at either site blocks catalysis. These findings are, in general, similar to those found previously in this laboratory using vanadate as inhibitor (Senior et al., 1995a; Urbatsch et al., 1995a,b), and they give strong confirmation of the concepts developed in that previous work.

In myosin, BeF_x inhibits ATPase by trapping MgADP in the catalytic site, forming the myosin·MgADP·BeF_x complex, the structure of which has been deduced by X-ray crystallography (Fisher et al., 1995). Notably, the complex shows tetrahedral geometry around the Be atom, which is in the position thought to be occupied by the γ-phosphorus atom in bound MgATP, and the Be to bridge-oxygen distance is the same as the γ-P to bridge-oxygen distance in MgATP. Thus the myosin·MgADP·BeF_x complex resembles the prehydrolysis state of bound MgATP. In contrast, the myosin·MgADP·V_i complex shows the structure expected

for the catalytic transition state, because it has trigonal bipyramidal geometry around the pentacovalent vanadium and the V atom to bridge-oxygen distance is longer than the equivalent bond in MgATP (Smith & Rayment, 1996). Thus it might be expected that the inhibited Pgp·MgADP·BeF_x complex would differ in detail from that of the Pgp·MgADP·V_i complex.

While inhibition of Pgp by BeF_x did resemble V_i inhibition in major characteristics, there were also differences. Notably, PP_i protected effectively against BeF_x inhibition of Pgp ATPase (this work) by competing with BeF_x whereas PP_i had no effect on inhibition by V_i (Urbatsch et al., 1995a). Here it was shown that 1 mM PP_i gave complete protection against BeF_x inhibition, and the apparent $K_i(PP_i)$ was 18 μM. P_i also protected against BeF_x inhibition, with apparent $K_i(P_i)$ of 3.2 mM. In previous work (Urbatsch et al., 1995b) we showed that P_i protected against V_i inhibition; however, comparison shows that P_i is ~10-fold more effective against BeF_x.

In summary, this work has demonstrated that beryllium fluoride (BeF_x) is a potent inhibitor of Pgp ATPase activity that tenaciously traps nucleotide in a catalytic site. The inhibited complex is a Pgp·MgADP·BeF_x species, and as in myosin, it appears to be nonidentical to the inhibited species generated by vanadate. BeF_x in combination with 8-azido-ATP allows specific photolabeling of Pgp at very low concentration of the photoprobe. Therefore BeF_x appears to be a valuable additional inhibitory reagent for structural and functional studies of Pgp.

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