P-Glycoprotein Shows Strong Catalytic Cooperativity between the Two Nucleotide Sites†

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ABSTRACT: P-Glycoprotein (Pgp) (also known as multidrug-resistance protein) contains two nucleotide binding sites, both of which are catalytic ATPase sites. The covalent reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) reacts in catalytic sites, and full inactivation of ATPase activity occurs at a reaction stoichiometry of 1 mol of NBD-Cl/mol of Pgp. We show that, at reaction stoichiometry of ≤1 mol/mol, both nucleotide sites become labeled in relatively nonselective fashion. There is therefore strong interaction between the two nucleotide sites because (a) reaction of one site with NBD-Cl severely impedes reaction of reagent with the other site, and (b) reaction of one site inhibits steady-state ATPase, i.e. both sites are inhibited. Vanadate-trapping experiments revealed that when one nucleotide site was reacted with NBD-Cl, not even a single ATPase turnover event could occur in the other, intact, nucleotide site. The data demonstrate therefore that catalytic cooperativity between the two nucleotide sites in Pgp is extremely strong and mandatory for catalysis.

P-Glycoprotein (Pgp,[1] also known as multidrug-resistance protein) is a plasma-membrane-located glycoprotein which confers multidrug-resistance phenotype on cells by virtue of its ability to exclude cytotoxic drugs in an ATP-dependent manner. The most commonly-considered current hypothesis is that Pgp acts as an ATP-driven drug-export pump, although the mechanism is not understood in detail and appears to involve novel features, e.g., presentation of transport substrates to the pump from the membrane lipid phase and an unusually wide variety of compounds acting as transport substrates. Multidrug resistance is an important obstacle in treatment of human cancer, and there is considerable interest in the potential role of Pgp in rendering tumor cells resistant to chemotherapeutic drugs (*1*−*6*).

Pgp molecules from humans and rodents show similar amino acid sequences and are about 1280 residues in length, with two nucleotide binding sites (NBS). Pgp shows substantial ATPase activity in the absence of drugs ("basal activity"), which is further stimulated several-fold by drugs. Kinetic properties of the ATPase activity have been characterized (*7*, *8*), showing that the catalytic sites are of low affinity for MgATP ($K_M \sim 1$ mM) and low specificity for nucleotide. However, kinetic studies could not detect interactions between the two NBS. Covalent labeling studies using NEM and 8-azido-ATP demonstrated that both NBS bind MgATP with approximately similar affinity, and that reaction of either NBS with reagent prevents steady-state ATP hydrolysis (*9*−*11*). Point mutations introduced independently into the "Walker A" sequence of NBS1 or NBS2 prevented conferral of multidrug-resistance phenotype in cells (*12*) and steady-state ATP hydrolysis (*13, 14*). These data established that both NBS must be intact for normal function.

Urbatsch et al. (*15*) found that vanadate (Vi) inhibits Pgp−ATPase activity by tenaciously trapping MgADP at a catalytic site, forming the Pgp·MgADP·Vi complex. Trapping of nucleotide in just one NBS per Pgp molecule was sufficient to block steady-state hydrolysis of MgATP, showing that the two NBS cannot function independently as catalytic sites. Subsequently, in Urbatsch et al. (*16*), trapping of nucleotide at NBS1 or NBS2 was seen to occur nonselectively, both NBS were shown to be capable of nucleotide hydrolysis, and trapping of nucleotide at either site prevented hydrolysis at both sites. This work provided clear evidence of mandatory, strong interaction between the two NBS in steady-state catalysis. Based on the above information, a model was proposed to explain the ATP-driven drug-pumping action of Pgp that involves a catalytic cycle in which the two NBS act alternately to hydrolyze MgATP (*17*).

The covalent inhibitor NBD-Cl presents opportunities to study further aspects of nucleotide site interactions in Pgp. In plasma membranes from the multidrug-resistant Chinese hamster ovary cell line CR1R12, the reaction of NBD-Cl with Pgp was remarkably specific (*9*). It reacted in catalytic sites of both membrane-bound and purified Pgp, and at a covalent labeling stoichiometry of 1 mol of NBD-Cl per mol of Pgp it fully inhibited steady-state ATPase activity (*9*, *18*). The following pertinent questions present themselves. Does NBD-Cl react with one NBS preferentially or with either NBS nonselectively? Does covalent labeling with NBD-Cl prevent nucleotide binding to one or both NBS? Does covalent labeling of just one NBS prevent MgATP hydrolysis entirely, or is there a single hydrolytic turnover, or perhaps

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[1] Abbreviations: Pgp, P-glycoprotein; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; Vi, orthovanadate; NBS, nucleotide binding site(s); MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid.

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a very slow rate of turnovers, in the unreacted NBS? Here we present experiments designed to study the interactions between Pgp nucleotide sites, using NBD-Cl as a tool.

MATERIALS AND METHODS

Preparation of Plasma Membranes. Plasma membranes were prepared from the multidrug-resistant Chinese hamster ovary cell line CR1R12 (*9*) as described previously (*15*). The membranes contained from 15 to 20% (w/w) of Pgp as a fraction of total membrane protein. Before use, the membranes were rendered DTT-free by elution through 1 mL centrifuge columns of Sephadex G-50 (fine) (*19*) equilibrated in 40 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, at a protein concentration of 2 mg/mL. DTT-free membranes were stored at −70 °C under N_2.

Assay of Pgp−ATPase Activity. ATPase activity was assayed using an ATP-regenerating system as described previously (*15*). An amount of 10 μg or less of membrane protein was added to 1 mL of assay medium at 37 °C 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 per minute per milligram of membrane protein.

NBD-Cl-Induced Inhibition of Pgp. Plasma membranes (50 μg of protein/100 μL) were incubated with varying concentrations of NBD-Cl in 40 mM Tris-HCl, pH 7.4, 0.1 mM EGTA at 37 °C for 10 min. NBD-Cl was added from stock solutions made in dimethyl sulfoxide and stored at −20 °C. Dimethyl sulfoxide concentration in the incubation was ≤1.4% (v/v). Concentration of NBD-Cl was calculated using the extinction coefficient of 1.15×10^4 M^{-1} cm^{-1} at 344 nm in H_2O at pH 7.1. The incubations were started by addition of membranes and stopped by passage of 100 μL samples through centrifuge columns consisting of 1 mL of Sephadex G-50 (fine) (*19*) equilibrated with 40 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, at 23 °C. Eluates were assayed for ATPase as described above.

Determination of Stoichiometry of [^{14}C]NBD-Cl Reaction with Pgp in Plasma Membranes. Plasma membranes were reacted with [^{14}C]NBD-Cl (35 dpm/pmol) and passed through centrifuge columns as described above. Samples (15−20 μg of protein) were run on SDS gels (without DTT), and gels were stained with Coomassie Blue. The Pgp band was excised, and radioactivity in Pgp was quantitated as described by Al-Shawi and Senior (*9*). The amount of Pgp in the membranes was analyzed as described by Al-Shawi et al. (*10*). 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 (*10*). The molecular size of Pgp was taken as 141 kDa.

Cleavage of Pgp in Plasma Membranes into N- and C-Terminal "Halves" by Mild Trypsin Digestion. The procedure followed that of Georges et al. (*20*). Plasma membranes were digested with trypsin (0.05 μg per 20 μg of plasma membrane protein) at 37 °C for 10 min, then SDS was added, and the digested samples were run on SDS gels. Where [^{14}C]NBD-Cl-labeled samples were run, DTT was omitted from the gels. The positions of the glycosylated N-terminal fragment (apparent mass = 100 kDa) and C-terminal fragment (apparent mass = 65 kDa) were verified by immunoblotting with C219 monoclonal antibody. Both could be seen also in Coomassie Blue-stained gels. Bands corresponding to the two halves of Pgp were excised from stained gels, and radioactivity was counted as described by Al-Shawi and Senior (*9*) (for ^{14}C) or by Cerenkov counting (for ^{32}P).

Vanadate-Induced Trapping of Nucleotide in Pgp Catalytic Sites. The procedure was as described by Urbatsch et al. (*15*). Briefly, samples that had been reacted with NBD-Cl and passed through centrifuge columns as above were incubated in 100 μL volume with 0.2 mM sodium orthovanadate, 2 mM $MgSO_4$, and MgATP (1 mM) or Mg-8-azido-ATP (50 μM), at 37 °C for 20 min. The samples were then passed through centrifuge columns consisting of 1 mL of Sephadex G-50 (fine) topped with a 10 mm layer of Dowex AG1-X8 (Bio-Rad) (*19*, *21*) equilibrated with 40 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, at 4 °C. Samples were assayed for ATPase activity as described above.

Vanadate-Trapping of Mg-8-azido[α-^{32}P]ATP Followed by UV Irradiation To Induce Covalent Labeling by Trapped Nucleotide. Vanadate-trapping experiments were done as above, using Mg-8-azido[α-^{32}P]ATP (50 μM, 750 dpm/pmol). Samples of the centrifuge column eluates were subjected to UV irradiation for 2 min on ice (λ = 254 nm, 1.1 mW/cm^2). Samples (20 μg of protein) were applied directly to SDS gels or were subjected first to mild trypsin digestion, as above, then applied to SDS gels. For quantitation of covalently bound radioactivity, the Pgp band or the fragments were excised from stained gels and counted by Cerenkov counting.

Direct Covalent Labeling of Pgp Using Mg-8-azido[α-^{32}P]ATP. Plasma membranes (50 μg of protein/100 μL) in 40 mM Tris-Cl, pH 7.4, 0.1 mM EGTA, 2 mM $MgS0_4$, at 23 °C were incubated with Mg-8-azido[α-^{32}P]ATP (10−200 μM, 750 dpm/pmol) for 30−60 s, then subjected to UV irradiation for 2 min on ice (λ = 254 nm, 1.1 mW/cm^2). Samples (20 μg of protein) were run on SDS gels, and radioactivity in the Pgp band was quantitated as above.

Routine Procedures. SDS gel electrophoresis, immunoblotting, and protein assay by bicinchoninic acid method in presence of 1% SDS were performed as in Al-Shawi and Senior (*9*).

Materials. 8-azido[α-^{32}P]ATP and [U-^{14}C]NBD-Cl were from Research Products International. NBD-Cl was from Sigma. Tissue culture materials were from BRL Life Technology Inc. Lactate dehydrogenase (catalog no. 127868) and pyruvate kinase (catalog no. 109053), both supplied in 50% glycerol, were from Boehringer Mannheim. C219 anti-Pgp monoclonal antibody was from Signet Laboratories.

Table 1: Inhibition and Labeling of P-Glycoprotein by NBD-Cl[a]

[NBD-Cl] (μM)	ATPase inhibition[b] (%)	[^{14}C]NBD-Cl[b] (mol/mol of Pgp)	distribution (%) of [^{14}C][c]	
			N-half	C-half
10	24	0.24	44	56
25	60	0.59	42	58
70	90.5	0.96	40	60

[a] Inhibition of ATPase activity by NBD-Cl, stoichiometry of labeling by [^{14}C]NBD-Cl, and distribution of covalently-bound label between N-terminal and C-terminal halves of Pgp were measured as in Materials and Methods. [b] Each experiment was performed at least in duplicate. [c] Each experiment was performed six to eight times.

RESULTS

Inhibition of ATPase Activity and Labeling of the Two Nucleotide Sites of Pgp by NBD-Cl. In this work we studied Pgp in plasma membranes from the multidrug-resistant Chinese hamster ovary cell line CR1R12. Previous work has shown that Pgp isoform-1 is the predominant isoform present (>95%) in these membranes (10). Chinese hamster Pgp isoform-1 is analogous to human multidrug-resistance protein isoform-1 (*mdr1*), which is responsible for multidrug resistance in human cancer (*2*). We first confirmed that ATPase activity of Pgp in the plasma membranes was potently inhibited by NBD-Cl and that full inhibition occurred at a stoichiometry of 1 mol of [^{14}C]NBD-Cl reacted/mol of Pgp. Table 1 presents these data, which in essence repeat earlier work (see Figures 7 and 9 of ref *9*), but were carried out here at a 10-fold higher protein concentration in order to allow further experiments on the NBD-Cl-reacted samples. Samples that had been reacted with [^{14}C]NBD-Cl to produce varying stoichiometry of labeling were subjected to mild trypsin proteolysis to cleave the Pgp into N- and C-terminal halves, containing NBS1 and NBS2, respectively, as was established by Georges et al. (*20*) using monoclonal antibodies with known epitopes. (An example of application of this technique to plasma membranes from CR1R12 cells in this laboratory may be found in ref 16.) Bands corresponding to the N- and C-terminal fragments were excised from gels and counted. The distribution of the radioactivity between them is shown in Table 1. It is evident that NBD-Cl reacts with either of the two NBS, with a small preference for the C-terminal.[2] This is an interesting result because it shows that reaction of either of the two NBS in a Pgp molecule with the covalent label prevents steady-state ATPase activity. Neither NBS can continue to carry out multiple turnovers of MgATP hydrolysis after the other site is labeled. It is reminiscent of the results obtained previously with Vi (see introductory section) and emphasizes the strong interaction between the two NBS in Pgp catalysis. Also, it is apparent that covalent reaction of NBD-Cl in one NBS strongly impeded reaction in the other NBS, at least under the conditions used in these experiments (higher NBD-Cl concentrations were not tested). This is further evidence of strong site−site interaction.

[2] We had earlier proposed that NBD-Cl reacted primarily with the C-terminal NBS of Pgp (I. L. Urbatsch and A. E. Senior, unpublished data quoted in ref *10*). This was based on visual inspection of fluorograms of trypsin-cleaved, [^{14}C]NBD-Cl-labeled Pgp. Subsequent experience has shown that due to the fact that the N-terminal, glycosylated, fragment of Pgp runs as a diffuse band on SDS gels, visual inspection of fluorograms leads to underestimation of labeling in the N-terminal NBS.

Table 2: Vanadate-Induced Inhibition of P-Glycoprotein ATPase Activity[a]

[NBD-Cl] (μM)	ATPase after NBD-Cl only (% inhibition)	ATPase after NBD-Cl then Vi plus ATP (% inhibition)	ATPase after NBD-Cl then Vi plus 8-azido-ATP (% inhibition)
0	0	94	85
10	28	90	84
25	60	91	89
70	90.5	92	95

[a] In a first incubation, NBD-Cl was added to Pgp in plasma membranes, and ATPase activity was assayed. In a second incubation, samples from the first incubation were subjected to the vanadate-trapping procedure using either Vi plus 1 mM MgATP or Vi plus 50 μM Mg-8-azido-ATP, and ATPase activity was assayed again. For details, see Materials and Methods.

Vanadate-Induced Trapping of Nucleotide in NBD-Cl-Reacted Pgp. It was determined previously (*15*, *16*) that Vi inhibits Pgp−ATPase activity by binding to the Pgp·MgADP species generated in the ATPase reaction, forming the noncovalent, but stable, inhibited Pgp·MgADP·Vi species, as follows:

$$\text{Pgp} + \text{MgATP} \rightarrow \text{Pgp}\cdot\text{MgATP} \rightarrow \text{Pgp}\cdot\text{MgADP}\cdot\text{P}_i \rightarrow \text{Pgp}\cdot\text{MgADP} + \text{P}_i \quad (1)$$

$$\text{Pgp}\cdot\text{MgADP} + \text{Vi} \rightarrow \text{Pgp}\cdot\text{MgADP}\cdot\text{Vi} \quad (2)$$

It was also demonstrated previously that Mg-8-azido-ATP substituted well as substrate for MgATP, producing the stable, inhibited species Pgp·Mg-8-azido-ADP·Vi, which on UV irradiation became permanently inactivated due to covalent attachment of 8-azido-ADP to the protein. The vanadate-trapping technique therefore allows a test of whether either of the NBSs in Pgp is catalytically-active. If Pgp is incubated with Mg-8-azido[α-^{32}P]ATP and vanadate, then subjected to UV illumination, covalent labeling of one or other of the NBS by the nucleotide will ensue only if at least one hydrolytic turnover event has occurred.

Here we first incubated Pgp in plasma membranes with NBD-Cl in order to achieve varying degree of inhibition of ATPase activity, up to ~90%, then subjected these samples to the vanadate-trapping procedure with MgATP or Mg-8-azido-ATP. Table 2 shows the initial degree of inhibition of ATPase activity caused by the first incubation with NBD-Cl (column 1) and the final degree of inhibition of ATPase activity after the subsequent vanadate-trapping procedure (columns 2 and 3). The data show that after partial inhibition by NBD-Cl, the remaining active molecules of Pgp may be fully inhibited by vanadate-trapping of nucleotide.

Next we took samples of Pgp in plasma membranes, incubated them first with NBD-Cl in order to achieve varying degree of inhibition of ATPase activity, then subjected these samples to vanadate-trapping in the presence of Mg-8-azido-[α-^{32}P]ATP, followed by UV irradiation and SDS gel electrophoresis. Figure 1 is an autoradiogram which shows labeling of Pgp by the vanadate-trapped Mg-8-azido[α-^{32}P]-ADP.[3] It is evident from comparing Figure 1 and Table 1 that the degree of vanadate-trapping of nucleotide was

[3] The lower band in Figure 1 (arrow) was shown to be a proteolytic fragment of Pgp (see Figure 1 legend). It is more evident than usual because these experiments required two consecutive incubations of plasma membranes at 37 °C, for 10 and 20 min, respectively.

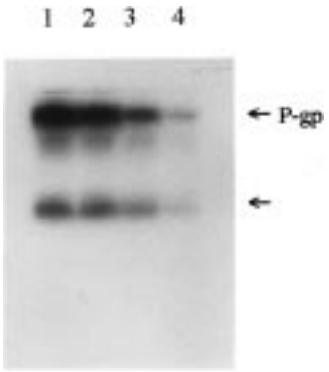

FIGURE 1: Reaction of Pgp with NBD-Cl, followed by vanadate-trapping with Mg-8-azido[α-^{32}P]ATP. Pgp in plasma membranes was reacted with NBD-Cl and passed through centrifuge columns. Eluates were then subjected to vanadate-trapping with 0.2 mM orthovanadate and 50 μM Mg-8-azido[α-^{32}P]ATP, passed through centrifuge columns, subjected to UV irradiation, and run on SDS gels. This is an autoradiogram of a typical gel. Lanes 1−4, pre-reaction with 0, 10, 25, and 70 μM NBD-Cl, respectively. The lower band (arrow) is a proteolytic product of Pgp, as determined by immunoblotting by C219 antibody, which is enhanced in this experiment due to the extended incubations of the plasma membranes at 37 °C.

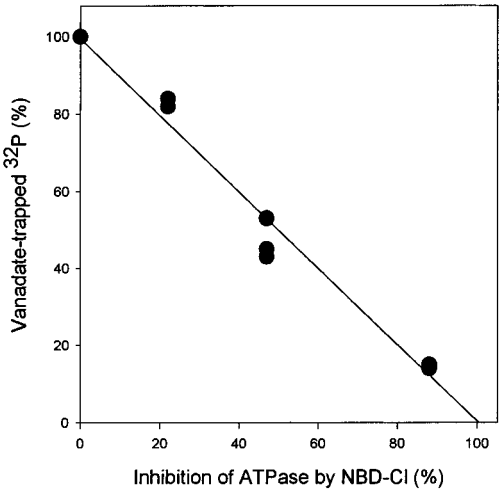

FIGURE 2: Inhibition of Pgp ATPase activity by NBD-Cl, followed by vanadate-trapping with Mg-8-azido[α-^{32}P]ATP. In the first incubation, Pgp in plasma membranes was reacted with varying concentration of NBD-Cl, passed through centrifuge columns, and assayed for ATPase activity. In the second incubation, samples of the NBD-Cl-inhibited Pgp were exposed to vanadate-trapping with 0.2 mM orthovanadate and 50 μM Mg-8-azido[α-^{32}P]ATP, passed through centrifuge columns, and subjected to UV irradiation and then SDS gel electrophoresis. Pgp bands were excised from stained gels and counted. For details, see Materials and Methods.

approximately proportional to the ATPase activity that remained after NBD-Cl inactivation. In samples that had been almost fully inhibited by NBD-Cl, very little vanadate-trapping of radioactive nucleotide occurred (Figure 1, lane 4).

Further experiments were carried out in which labeling of Pgp by vanadate-trapped Mg-8-azido[α-^{32}P]ADP after UV irradiation was quantitated by excising the Pgp band from gels and counting the radioactivity. In Figure 2 these data are plotted against the residual ATPase activity remaining after NBD-Cl inhibition measured in the same experiments. It is seen that as residual ATPase activity declined to zero, so did the amount of vanadate-trapped nucleotide. The data in Figures 1 and 2 establish that in a Pgp molecule which is covalently reacted with NBD-Cl in just one of the two NBS, not even a single ATP hydrolysis turnover event occurs in either of the two NBS.

Samples that had been reacted with NBD-Cl, then subjected to vanadate-trapping with Mg-8-azido[α-^{32}P]ATP and UV irradiation as in Figure 1, were cleaved by mild trypsin proteolysis into N- and C-terminal fragments, then run on gels, in order to test whether reaction with NBD-Cl altered the relative distribution of vanadate-trapping of nucleotide in the two NBS. The distribution of covalently-bound ^{32}P between the two fragments (N-terminal/C-terminal ratio) was as follows: 0 NBD-Cl, N/C ratio = 65/35; 10 μM NBD-Cl, 65/35; 25 μM NBD-Cl, 63/37; 70 μM NBD-Cl, 65/35 (means of quadruplicate experiments). Reaction with NBD-Cl did not therefore significantly affect the relative distribution of vanadate-trapped nucleotide, consistent with the idea that in NBD-Cl-reacted Pgp molecules neither NBS is catalytically functional.

Direct Covalent Labeling of NBD-Cl-Inhibited Pgp by Mg-8-azido[α-^{32}P]ATP. If NBD-Cl reaction eliminated nucleotide binding to both of the Pgp NBS, this would provide a straightforward but trivial explanation for the inhibition of catalysis seen above. Thus it was important to determine whether this was the case. Mg-8-azido-ATP is known to be an excellent hydrolysis substrate for Pgp (*10*). Direct covalent labeling of Pgp in plasma membranes by Mg-8-azido[α-^{32}P]ATP was therefore carried out. Preliminary experiments established that, as has been shown previously in this and many other laboratories, covalent labeling of Pgp by Mg-8-azido[α-^{32}P]ATP was readily apparent on autoradiograms of SDS gels, and the presence of excess MgATP (2 mM) abolished labeling (data not shown). It was also confirmed, using mild trypsin proteolysis, that Mg-8-azido-[α-^{32}P]ATP reacted relatively equally in both of the Pgp nucleotide-binding sites, as shown previously (*10*, *20*). In order to quantitate the covalent labeling, radioactive nucleotide was incubated directly with NBD-Cl-reacted Pgp in plasma membranes, samples were subjected to UV irradiation and then run on SDS gels, the Pgp band was excised, and covalently-bound ^{32}P was counted, as described in Materials and Methods.

The stoichiometry of covalent 8-azido[α-^{32}P]ATP labeling of Pgp in plasma membranes in control samples (which were preincubated in absence of NBD-Cl) is shown in column 2 of Table 3. The K_M(Mg-8-azido-ATP) is 0.5 mM (*10*). On the assumption that the average degree of occupancy of the Pgp nucleotide sites is 1 mol/mol of Pgp at the K_M concentration, the data in column 2 of Table 3 indicate that the efficiency of covalent labeling was ∼50%, which is relatively high. Increasing the duration of UV illumination did not increase the degree of labeling. This was expected as previous work has shown that all the 8-azido-ATP would be photoactivated under the conditions used (*22*).[4]

The effect of preincubation of Pgp with NBD-Cl is shown in columns 3−5 of Table 3, the data being tabulated as % effect (+/−) on the Mg-8-azido[α-^{32}P]ATP labeling stoi-

[4] Higher labeling stoichiometries (1−2 mol/mol of Pgp) can be achieved with 8-azido[α-^{32}P]ATP if several consecutive incubations with high concentration of label are used (*10*). This is economically not feasible for multiple experiments.

Table 3: Direct Covalent Labeling of Pgp in Plasma Membranes by Mg-8-azido[α-^{32}P]ATP[a]

[8-azido-ATP] (μM)	stoichiometry of labeling[b] (mol of 8-azido-ATP/mol of Pgp)	effect of preincubation with NBD-Cl on 8-azido-ATP labeling (% change in stoichiometry)		
		10 μM NBD-Cl	25 μM NBD-Cl	70 μM NBD-Cl
10	0.008 (2)[b]	0 (2)	+5 (2)	+11 (2)
25	0.024 (4)	+11 (3)	+13 (3)	−15 (4)
50	0.056 (6)	+14 (2)	+7 (2)	−6 (6)
100	0.108 (4)			−13 (4)
200	0.195 (2)			−12 (2)

[a] In a first incubation, NBD-Cl was added to Pgp in plasma membranes in order to bring about covalent labeling and inhibition of ATPase activity, and the membranes were eluted through centrifuge columns to remove unreacted NBD-Cl, as described in Materials and Methods. $MgSO_4$ (final concentration = 2 mM) and Mg-8-azido[α-^{32}P]ATP (concentration shown) were added, the tubes were mixed and incubated for 30−60 s at room temperature, and they were then placed on ice and subjected to UV illumination (see Materials and Methods). Finally samples were run on SDS gels (20 μg of protein per lane), the Pgp band was excised after staining, and the radioactivity was counted. [b] This column shows labeling of control membranes which were preincubated without NBD-Cl, then subjected to covalent labeling with Mg-8-azido[α-^{32}P]ATP. [c] Number of replicates is in parentheses.

chiometry. It is evident that even at the highest concentration of NBD-Cl there were only small effects on covalent labeling by Mg-8-azido[α-^{32}P]ATP. It was confirmed that the distribution of the covalent Mg-8-azido[α-^{32}P]ATP label between the two NBS was not altered by preincubation with NBD-Cl. It is evident that the reduction in ATPase activity (Table 1) and the reduction of vanadate-trapping of nucleotide (Figures 1 and 2) cannot be explained as due simply to reduction of nucleotide binding to the catalytic sites.

It is in fact interesting that the effects seen in Table 3 are so small. Since MgATP protects Pgp from reaction with NBD-Cl (*9*, *18*), one might have expected that pre-reaction with NBD-Cl would prevent nucleotide binding in at least one of the NBS. However, Liu and Sharom (*23*, *24*) have shown that labeling of Pgp at specific Cys residues within the Walker A sequences of the catalytic sites by the large fluorescent probe 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS) is prevented by MgATP; but if Pgp is first reacted with MIANS, the catalytic sites can subsequently bind MgATP. This phenomenon is presumably due to the fact that the Pgp catalytic sites are unusually conformationally flexible (*7*). It may also be noted that mutagenesis of the Lys residues in the Walker A sequences of the Pgp catalytic sites has no significant effect on covalent binding of Mg-8-azido[α-^{32}P]ATP (*12*, *14*). Therefore, there are precedent examples where alteration of Pgp catalytic site structure leads to loss of catalysis without apparent significant impairment of nucleotide binding.

DISCUSSION

NBD-Cl is a potent inactivator of Pgp−ATPase activity which reacts in catalytic sites and yields full inactivation on covalent reaction of 1 mol NBD-Cl/mol of Pgp (*9*, *18*). Here we demonstrate that at labeling stoichiometry of ≤1 mol/mol, both nucleotide sites (NBS) of Pgp are labeled by NBD-Cl in relatively nonselective fashion. Therefore, covalent reaction of either NBS in a molecule of Pgp is sufficient to block steady-state hydrolysis, confirming that neither site hydrolyzes MgATP independently and emphasizing the requirement for interaction between the two NBS for catalysis. Further, the fact that reaction of one NBS with NBD-Cl severely impedes reaction of the other NBS is also evidence of strong site−site interaction.

Vanadate-trapping experiments provide a powerful tool for studies of Pgp cataytic sites. For example, as was detailed in Results, when used in conjunction with 8-azido-[α-^{32}P]ATP, vanadate-trapping can demonstrate whether hydrolytic turnover is occurring in either of the two NBS. Here it was found that in molecules of Pgp that were inhibited by NBD-Cl prior to vanadate-trapping, zero ATP hydrolysis turnover occurred in either of the two NBS. This shows that there is extremely strong catalytic cooperativity in Pgp, such that inactivation of one NBS prevents even a single turnover of MgATP hydrolysis from occurring in the other, intact NBS.

A straightforward explanation for this result could, however, be that reaction of Pgp with NBD-Cl prevents nucleotide binding to the NBS, or at least decreases the affinity of the NBS to such a degree that significant nucleotide binding no longer occurs under the experimental conditions used. Attempts to directly measure nucleotide binding to Pgp by equilibrium methods have so far not been successful due to the low apparent affinity of the catalytic sites and the particulate nature of the available experimental materials (*15*). Therefore we used here an indirect approach, namely covalent labeling of Pgp by added Mg-8-azido-ATP. Because covalent insertion of the label may occur to differing extent in the two NBS (irrespective of their binding affinities) and differential losses of label may occur during subsequent manipulations such as centrifuge column elution and SDS gel electrophoresis, the results must be interpreted with caution. We found however (Table 3) that in NBD-Cl-inhibited Pgp there was no significant effect on covalent labeling by 8-azido-ATP, as compared to the native Pgp control. It is justified therefore to conclude that the inhibition of catalysis by NBD-Cl is not simply a consequence of impaired substrate binding to the NBS.

The enzyme F_1-ATPase may provide a useful parallel to Pgp. It is known that NBD-Cl inhibits ATPase activity of F_1-ATPase when it covalently reacts within the catalytic site, and full inhibition of this enzyme is obtained when just one of the three catalytic sites is reacted with NBD-Cl (*25*−*28*). Sequential, cyclical involvement of each of the three catalytic sites of F_1-ATPase in steady-state ATP hydrolysis has now been proven (*29*, *30*). The results presented here with Pgp point strongly to a sequential sites mechanism for Pgp ATPase, in which each of the two NBS participates alternately in catalysis, as previously proposed (*17*).

In summary, we conclude that after reaction and inactivation of one NBS of Pgp by NBD-Cl, not even a single

hydrolytic turnover of bound substrate occurs in the other NBS. Catalytic cooperativity between the two catalytic sites is an integral feature of Pgp function.

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