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ATP Hydrolysis by Multidrug-Resistance Protein from Chinese Hamster Ovary Cells

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ATPase activity of multidrug-resistance protein (P-glycoprotein, Pgp) from Chinese hamster ovary cells was studied. Catalytic characteristics were established for Pgp both in its natural plasma membrane environment and in purified reconstituted protein. Generally the two preparations of Pgp behaved similarly, and demonstrated low affinity for MgATP, low nucleotide specificity, preference for Mg-nucleotide, and pH optimum near 7.5. A high-affinity binding site involved in catalysis was not apparent. Effective covalent inactivators were NBD-C1, NEM, 8-azido-ATP, and 2-azido-ATP. DCCD, FITC, and pyridoxal phosphate were only weakly inhibitory. Lipid composition was found to affect the degree of drug stimulation of ATPase in purified reconstituted Pgp, suggesting that the lipid environment affects coupling between drug-binding and catalytic sites, and that Pgp expressed in different tissues could show different functional characteristics.

KEY WORDS: MDR protein; ATP hydrolysis; catalytic sites.

INTRODUCTION

Our interest in multidrug-resistance protein (Pgp)[2] was aroused when the first published amino acid sequences revealed the presence of two "Walker A and B" consensus sequences (Walker *et al.*, 1982), indicating that the protein contained at least two nucleotide sites. Pgp[3] is a plasma membrane-located protein, and topological models suggest that it consists of two duplicated "halves," each with a nucleotide site contained in a membrane-extrinsic, cytoplasmic-sided domain (Gottesman and Pastan, 1993). Biochemical evidence derived from studies of multidrug-resistant cells (Bradley *et al.*, 1988; Gros *et al.*, 1992) has shown that Pgp is a membrane transporter which couples ATP hydrolysis to drug extrusion from cells, and provides a functional rationale for the observations linking Pgp to resistance of human cancers to chemotherapy. This raises important opportunities.

Techniques such as (photo)affinity labeling, site-directed mutagenesis, fluorescence spectroscopy, and kinetic analysis have already been applied successfully in studies of ATPase enzymes. Methods to inhibit ATPases through use of covalent or noncovalent reagents are well known. Structures of nucleotide-binding sites in several enzymes have been established by X-ray crystallography at high resolution. Thus, there is a wealth of existing experimental and theoretical information which we can draw upon to expedite studies of catalysis in Pgp. Here we briefly summarize the work from our laboratory on this topic.

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[2] Multidrug-resistance protein is also called P-glycoprotein. We use the abbreviation Pgp in this paper.

[3] Abbreviations: Nucleotide analogs: *lin*-benzo-ATP, 8-amino-3-(β-D-ribofuranosyl)imidazo[4,5-*g*]quinazoline-triphosphate (contains formally a benzene ring inserted between pyrimidine and imidazole rings of adenosine, linearly extending the ring system); AMPPNP, adenyl-5′-yl-imidodiphosphate; TNP-ATP, 2′,3′-*O*-(2,4,6)-trinitrophenyl-ATP; BzATP, 3′-*O*-(4-benzoyl)benzoyl-ATP; ATPγS, adenosine-5′-*O*-(3-thiotriphosphate). *Other compounds*: DCCD, dicyclohexylcarbodiimide; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; NEM, *N*-ethylmaleimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; TPA$^+$, tetraphenylarsonium ion; TPP$^+$, tetraphenylphosphonium ion; Pgp, P-glycoprotein.

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SOURCE OF EXPERIMENTAL MATERIAL

Enriched membrane preparations containing a high density of pump molecules have proved extremely valuable for biochemical studies of ATP-driven transport proteins. Sarcoplasmic reticulum (Ca-ATPase), kidney membranes (Na-K-ATPase), and mitochondrial inner membrane (F_1F_0-ATP synthase) are three examples that come readily to mind. Indeed many features of these systems were already established from studies of enriched membranes before the advent of purification/reconstitution techniques. We considered it important therefore to devise a source from which plasma membranes enriched in Pgp could be obtained. The work of Kartner *et al.* (1985) pointed the way to this goal by showing that Chinese hamster ovary cells, selected stepwise for increased resistance to colchicine, overexpressed Pgp. Following essentially this procedure, we obtained a cell line (CR1R12) which grows well and constitutively overexpresses Pgp. CR1R12 cells were grown in suspension culture, disrupted by N_2-cavitation using a Parr apparatus, and plasma membranes were isolated by sucrose gradient centrifugation, carried out essentially as described by Riordan and Ling (1979). The resultant plasma membrane preparation was found to contain a high level of Pgp (average = 21% of total membrane protein; Al-Shawi and Senior, 1993). In a recent modification of this procedure we have substituted a Bio-Neb Cell Disrupter (Glas-Col Co., Terre Haute, Indiana) for the Parr apparatus, with considerable increase in yield of plasma membranes.

The enriched plasma membrane preparation provided a good starting point for purification of Pgp. Purification was accomplished in a three-step procedure consisting of (1) octylglucoside solubilization in the presence of *E. coli* lipids and glycerol (Ambudkar *et al.*, 1992); (2) Reactive Red 120 agarose chromatography in the presence of *E. coli* lipids, glycerol, and a high concentration of ATP (50 mM); (3) reconstitution in proteoliposomes. The procedure is described in detail by Urbatsch *et al.* (1994).

In both purified reconstituted Pgp and enriched plasma membranes, isoform Pgp-1 accounted for >95% of the total Pgp (Al-Shawi *et al.*, 1994, Urbatsch *et al.*, 1994), as determined using monoclonal antibodies C219 and C494 (Georges *et al.*, 1990). Chinese hamster Pgp-1 is the isoform analogous to human mdr1, which is responsible for resistance to cancer chemotherapy (Gottesman and Pastan, 1993).

Possibly the purification scheme devised here may be applicable to Pgp expressed in other membrane systems, e.g., *E. coli* (Bibi *et al.*, 1993), yeast (Ruetz and Gros, 1994), or insect (Sarkadi *et al.*, 1992). Studies of Pgp would certainly be accelerated by discovery of a more plentiful and less expensive source of material than is currently available; for structural resolution this will be a *sine qua non*.

CATALYTIC CHARACTERISTICS OF PGP

Enriched Plasma Membranes from CR1R12 cells

There was substantial ATPase activity in the plasma membranes which was stimulated up to 5-fold by verapamil, and 1.6- to 1.9-fold by colchicine, vinblastine, or daunomycin (Al-Shawi and Senior, 1993). The (low) Na,K-ATPase activity could be eliminated by inclusion of 2 mM ouabain in assays, and the (very low) Ca-ATPase activity by inclusion of EGTA. Mitochondrial and ecto-ATPase activities were negligible. In the presence of ouabain and EGTA, the total ATPase activity correlated well with membrane Pgp content. The enriched plasma membrane preparation from CR1R12 cells therefore provided a system in which to characterize catalytic properties of the Pgp embedded in its natural lipid environment.

Basic catalytic characteristics were established and are described in detail by Al-Shawi and Senior (1993). Summarizing these briefly, the Pgp ATPase turnover (in the presence of verapamil) was a respectable 21 s^{-1}; the optimal pH was 7.3; MgATP was the preferred substrate, with CaATP being poorly hydrolyzed; K_M (MgATP) = 1.4 mM, whereas K_i (MgADP) = 0.35 mM. GTP and ITP were real but poor substrates, and ADP and AMP were not hydrolyzed. Among noncovalent inhibitors, NaN_3 was ineffective, whereas vanadate and fluoroaluminate (both of which are potential pentacoordinate phosphorus transition state analogs in combination with ADP) were effective, with 50% inhibition occurring at 12 and 61 μM, respectively. K_i (MgAMPPNP) was unusually high at 0.44 mM.

It may be noted that the CR1R12 plasma membranes represent an excellent system for screening of potential "multidrug-reversal" agents by enzymatic assay.

Purified Reconstituted Pgp

The catalytic characteristics of purified Pgp reconstituted in *E. coli* lipid proteoliposomes were in

Table I. Specificity of P-glycoprotein for Mg-Nucleotides as Substrates and Inhibitors

Nucleotide	Plasma membrane Pgp			Purified reconstituted Pgp		
	V_{max}[a]	K_M[b]	k_{cat}/K_M[c]	V_{max}[a]	K_M[b]	k_{cat}/K_M[c]
ATP	9.0	1.4	1.5×10^4	3.9	0.8	1.2×10^4
2′dATP	6.7	1.1	1.4×10^4	2.0	1.1	4.3×10^3
2-azido-ATP	1.8	0.37	1.1×10^4	—	—	—
8-azido-ATP	3.6	0.50	1.6×10^4	1.2	0.5	5.6×10^3
8-bromo-ATP	—	—	—	1.1	1.3	2.0×10^3
1,N^6-etheno-ATP	—	—	—	2.7	2.0	3.2×10^3
lin-benzo-ATP	—	—	—	0.90	—	—
GTP	0.97	—	—	0.14	—	—
ITP	1.8	—	—	0.21	—	—
UTP	—	—	—	0.06	—	—
CTP	—	—	—	0.15	—	—
ADP	0	$K_i = 0.35$	—	0	$K_i = 0.70$	
AMP-PNP	0	$K_i = 0.44$	—	0	$K_i = 0.35$	
ATPγS	—	—	—	0	$K_i = 0.07$	
TNP-ATP	—	—	—	0	$K_i = 0.40$	
BzATP	—	—	—	0	$K_i = 0.45$	
AMP	0	$K_i = \gg 5.0$	—	0	$K_i \gg 10.0$	

[a] V_{max} in μmol/min/mg Pgp in presence of verapamil to give maximal stimulation. Pgp content of plasma membranes determined by laser densitometry/immunodetection as in Al-Shawi *et al.* (1994).
[b] K_M in mM (also K_i).
[c] k_{cat}/K_M in $M^{-1}s^{-1}$.

most instances very similar to those seen in the plasma membranes. For example, K_M (MgATP) = 0.8 mM and and $k_{cat} = 9.2\ s^{-1}$ (in the presence of verapamil); the pH dependence showed a broad optimum around pH 7.5; MgATP was greatly preferred over CaATP as substrate; and vanadate inhibited by 50% at 9 μM (Urbatsch *et al.*, 1994). Table I compares the nucleotide specificity of purified reconstituted Pgp with that of Pgp in plasma membranes.

Effect of Lipids

There was, however, one notable difference between purified reconstituted Pgp and Pgp in plasma membranes, involving the degree of stimulation of ATPase activity elicited by drugs and modulators. Verapamil stimulated ATPase activity by up to 5-fold in plasma membranes but by only 2-fold in purified reconstituted Pgp. Furthermore, vinblastine, daunomycin, and colchicine gave no stimulation of purified reconstituted Pgp. Titration studies showed that each of these compounds actually had a biphasic effect on Pgp ATPase activity in plasma membranes, with a stimulatory effect at lower drug concentrations, followed by an inhibition at higher concentrations. Interestingly, the inhibitory effect of vinblastine, daunomycin, and colchicine at higher concentrations was preserved in purified reconstituted Pgp, implying that there was interaction between the drugs and Pgp. This was supported by experiments in which it was seen that azidopine-labelling was also decreased in the presence of the drugs (Urbatsch and Senior, 1994).

Several other compounds were tested for stimulation of purified reconstituted Pgp ATPase activity, and did stimulate, including nifedipine, trifluoperazine, rhodamine-123, progesterone, dexamethasone, hydrocortisone, the peptides ALLN, leupeptin and pepstatin A, quinidine, the lipophilic cations TPP^+ and TPA^+, and the calcium probes fura-2AM and indo-1AM (the analogous free acids did not stimulate).

We suspected from comparison of our results with those of Shapiro and Ling (1994) that the degree of drug stimulation of ATPase activity in purified reconstituted Pgp might be affected by lipid composition. In order to examine the effects of lipids on purified Pgp, we first performed purification in the absence of added lipid. The material eluting from the Reactive Red 120 agarose chromatography column lacked ATPase activity, and no activity could be restored by addition of lipid, showing that the presence of lipids during purification is mandatory

Table II. Characteristics of ATPase Activity of P-glycoprotein Purified and Reconstituted in Different Lipids

	E. coli lipids	Sheep brain lipids	Bovine liver lipids
Basal ATPase V_{max} [a]	1.9	1.3	0.64
Basal ATPase K_M [a]	0.7 mM	0.9 mM	0.8 mM
Plus verapamil V_{max} [b]	4.1 (2.2 ×)	3.7 (2.9×)	2.3 (3.6 ×)
Plus verapamil K_M [b]	0.8 mM	0.7 mM	0.6 mM
Plus vinblastine[c] V_{max}	2.0 (1.0 ×)	1.9 (1.5 ×)	1.1 (1.7 ×)

[a] "Basal ATPase" is defined as the activity in assay buffer alone without added drug. V_{max} values are given as μmol ATP hydrolyzed/min/mg Pgp. Values in brackets are the degree of stimulation over basal level.
[b] 50 μM verapamil.
[c] Maximal values are given. With sheep brain lipids this was at 10 μM vinblastine, with bovine liver lipids at 5 μM vinblastine. No stimulation occurred in *E. coli* lipids at any vinblastine concentration.

to maintain integrity of Pgp. Next, we substituted crude sheep brain lipids or bovine liver lipids for the *E. coli* lipids during purification and reconstitution of Pgp. As Table II shows, this restored the stimulation of ATPase activity by vinblastine. Restoration of stimulation by daunomycin and colchicine was also seen (Urbatsch and Senior, 1994).

COVALENT INACTIVATORS OF PGP ATPase ACTIVITY

Several reagents were seen to inactivate ATPase activity of Pgp, namely NBD-Cl, NEM, 8-azido-ATP, and 2-azido-ATP. The characteristics of inhibition were similar in both plasma membrane and purified reconstituted Pgp (Al-Shawi *et al.*, 1994; Urbatsch *et al.*, 1994). Other reagents which have been seen previously to inactivate and label ATPase enzymes were found to be only weakly effective against Pgp, namely DCCD, FITC, and pyridoxal-5′-phosphate.

NBD-Cl inactivated Pgp potentialy in a DTT-irreversible and MgATP-protectable manner. Complete inactivation corresponded by extrapolation to incorporation of 1.1 mol NBD/mol Pgp, and trypsin hydrolysis (which splits Pgp into two "halves"; Georges *et al.*, 1991) showed that the label resided entirely in the C-terminal "half" of [^{14}C]NBD-labeled Pgp. The data are consistent with the idea that NBD-Cl is reacting at a catalytic site lysyl residue (rather than Cys or Tyr), located in the C-terminal nucleotide site.

NEM inactivated potentially in an MgATP-protectable manner. Complete inactivation corresponded by extrapolation to incorporation of ~2 mol NEM per mol Pgp. Trypsin hydrolysis revealed that the [^{14}C]NEM label was incorporated equally into N- and C-terminal "Halves" of Pgp, suggesting that both nucleotide sites were labelled. The kinetics and pH-dependence of the reaction indicates that cysteine was the target residue. $HgCl_2$ was also a potent inactivator. Interestingly, each of the two "Walker A" consensus sequences in Pgp contains a Cys residue.

Both 8-azido-ATP and 2-azido-ATP are excellent substrates for hydrolysis by Pgp (Table I), and on photoactivation both inhibited ATPase activity potently, with MgATP offering protection. 8-azido-ATP labelling has been studied in detail. Complete inactivation occurred concomitant upon incorporation of 2 mol 8-azido-ATP/mol Pgp, and the label was found equally distributed in the two "halves" of Pgp after trypsin cleavage (Georges *et al.*, 1991; Al-Shawi *et al.*, 1994). It thus appears that 8-azido ATP labels both predicted nucleotide sites.

Protein chemistry procedures (fragmentation, sequencing) are being applied to identify residues labelled by each of these four covalent labelling reagents.

CONCLUSIONS

1. ATP hydrolysis in Pgp occurs at an apparently low-affinity, low nucleotide-specificity catalytic site or site(s). MgATP is the physiological substrate. Relatively high K_M (MgATP) and K_i (MgADP) values suggest that drug transport rates could be sensitive to ATP depletion under some cellular conditions.

2. The high K_i (MgAMPPNP) and low k_{cat}/K_M (MgATP) suggest there is no high-affinity site involved

in catalysis. This could have significant mechanistic implications. Transport ATPases commonly display high-affinity ATP binding and/or covalent E ~ P intermediate species (e.g., F_1F_0-ATP synthase, vacuolar-ATPase, P-type ATPases). In currently discussed models of transport, changes in free energy associated with these species during the catalytic cycle are considered to be coupled by conformational signal transmission through the protein to changes of both binding affinity and "sidedness" at the transport substrate binding sites, allowing transmembrane transport and net release against an (electro)chemical gradient (e.g., Tanford, 1983). Lack of a high-affinity binding site for ATP, and the presumed (although not yet proven) absence of a catalytic intermediate E ~ P species in Pgp, would lead to consideration of different models, in which, for example, drug transport is coupled to bond cleaveage *per se*.

3. From the data available it is not possible to predict the number of catalytic sites in Pgp. There is a single K_M (MgATP), and MgADP and MgAMPPNP behave as classical competitive inhibitors. NBD-Cl gives full inactivation at ~1 mol/mol, whereas NEM and 8-azido-ATP react at two sites. One might speculate that two sites alternate in catalysis; or that one, unique, site is catalytically active, but only so when a second site is occupied by ATP. Since direct equilibrium binding studies with nucleotides or analogs have not yet been reported, we lack essential information to help decide this question. Previous mutagenesis studies of Azzaria *et al.* (1989) showed that mutagenesis within either of the two "Walker A" sequences inactivated Pgp when the Pgp was expressed in cultured mammalian cells. However, Bibi *et al.* (1993) reported that the "double Walker A" mutated Pgp was active when expressed in *E. coli* cells. Possibly a targeting defect could be responsible for inactivation of mutant Pgp expressed in mammalian cells. Loo and Clarke (1994) demonstrated that each "half" of Pgp, when expressed separately from the other half, had significant ATPase activity. Therefore, each nucleotide site has the potential capability for catalytic activity. While this topic may seem esoteric, cooperativity between nucleotide-binding sites has featured prominently in previously proposed functional models for ATP-driven membrane transporters, and would seem germane to understanding the mechanism of action of Pgp.

4. Use of the covalent inactivators NEM and NBD-Cl showed that there are probably reactive cysteine and lysine residues in the Pgp catalytic sites. Use of photoaffinity and affinity labelling probes promises to reveal more information regarding catalytic site architecture and reactive residues. It may be optimistic but it is not unrealistic to suggest that, based on this type of information, suicide inhibitors may be developed to specifically inhibit Pgp *in vivo*.

5. The lipid environment can affect apparent coupling between Pgp drug transport sites and ATPase sites. Specifically, the degree of stimulation of ATPase activity by drugs, e.g., verapamil and vinblastine, was different when Pgp was purified and reconstituted in different lipids. This raises the possibility that the same Pgp isoform expressed in different tissues could show different functional characteristics. Techniques used to date for studying interactions and coupling between catalytic sites and drug transport sites have been ATP-driven drug transport assays (e.g., Sharom *et al.*, 1993; Horio *et al.*, 1991; Ruetz and Gros, 1994), drug-stimulated ATPase assays (e.g., Rao and Scarborough, 1994), and nucleotide-induced modulation of azidopine-labelling (Rebbeor and Holohan, in preparation; Urbatsch and Senior, 1994). This aspect of Pgp function seems central to understanding Pgp mechanism and regulation.

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