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Sensitivity of P-glycoprotein tryptophan residues to benzodiazepines and ATP interaction

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Abstract

Plasma membrane P-glycoprotein is a member of the ATP-binding cassette family of membrane transporters. In the present study tryptophan intrinsic fluorescence was used to understand the P-glycoprotein response to three benzodiazepines (bromazepam, chlordiazepoxide and flurazepam) in the presence and absence of ATP. Fluorescence emission spectra showed a red shift on the maximal emission wavelength upon interaction of P-glycoprotein with all benzodiazepines. Benzodiazepine association with nucleotide-bound P-glycoprotein also showed this trend and the quenching profile was attributed to a sphere-of-action model, for static fluorescence. Furthermore, quenching data of benzodiazepine-bound P-glycoprotein with ATP were concentration dependent and saturable, indicating that nucleotide binds to P-glycoprotein whether drug is present or not. These results seems in agreement with the proposal of the ATP-switch model by Higgins and Linton, where substrate binding to the transporters initiates the transport cycle by increasing the ATP binding affinity.

Keywords: P-glycoprotein; Intrinsic fluorescence; Benzodiazepines; ATP; Interactions

1. Introduction

The P-glycoprotein multidrug transporter exports a wide range of unrelated molecules from the plasma membrane of eukaryotic cells, driven by the energy provided from ATP hydrolysis. According to its sequence analysis P-glycoprotein belongs to the ATP binding cassette (ABC) superfamily of membrane transport proteins. Like all ABC proteins P-glycoprotein is composed by two homologous halves, each comprising a transmembrane (TM) segment made of six membrane-spanning α-helices and a cytosolic nucleotide binding (NB) domain for nucleotide binding.

This efflux pump is present in the luminal surface of the endothelial cells of the brain cells and it seems to be responsible for the very poor penetration of many relatively large hydrophobic drugs in the brain, by performing active back-transport of these drugs to the blood [1]. It has been implicated in the resistance of human tumors to chemotherapeutic drugs [2,3] and is also known to play an important physiological role in preventing absorption of many drugs at the small intestine luminal surfaces [4]. However, while tumor drug resistance and drug oral bioavailability are quite well studied, there are few reports investigating the affinity of anxiolytics for P-glycoprotein. Benzodiazepines belong to a class of neuroactive drugs that are widely administrated due to their muscle relaxant, hypnotic, anti-convulsant and anxiolytic characteristics [5]. Recently, a P-glycoprotein inhibitor increased morphine concentration in rat brain extracellular fluid while chronic morphine treatment lead to tolerance of the antinociceptive effect associated with induction of brain P-glycoprotein [6,7]. This information suggests an important role of P-glycoprotein in regulating brain tissue access of centrally acting drugs and consequently in determining their pharmacokinetics effects.

Direct structural analysis of membrane proteins is difficult and, several indirect approaches have revealed some of the structure of Pgp [8,9]. In past few years, fluorescence spectroscopy has become a powerful tool for our understanding of the structure and function of membrane proteins, reviewed on [10–12]. A major advantage of intrinsic fluorescence is that the native protein can be directly used. Initially, fluorescence experiments were carried out

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on purified P-glycoprotein labelled at Cys with an extrinsic fluorophore (MIANS, [2-(4-maleimidoanilino)naphathalene-6-sulfonic acid]) [13,14], which indicated crosstalk between the catalytic sites of the protein and the drug binding, probably in the TM segments. Inactivation of the transporter catalytic activity was a major limitation, only overcome by the use of intrinsic fluorophores, like Trp residues. Mouse MDR3 Pgp contains 11 tryptophan (Trp) residues, 5 in the TM segments and 6 in the cytoplasmatic regions of the protein and, their position is highly conserved in both human (MDR1) and mouse (MDR3) homologues [15]. Pawagi et al. [16] suggested that Trp residues may be involved in substrate recognition and interaction, since these residues are over-represented in the TM segments and are quite conserved across the P-glycoprotein family. The first work on the intrinsic fluorescence of P-glycoprotein was conducted on Sonveaux group. Initially they have reported quenching of the P-glycoprotein Trp residues using iodide ion quenching [17] and later on acrylamide quenching as an indicator of conformational changes following binding of various anthracycline derivatives and ATP [18]. Later on Liu et al. reported a comprehensive study of the intrinsic fluorescence properties of catalytically active P-glycoprotein by steady state and lifetime fluorescence experiments using unmodified and fluorescent derivatives of nucleotides and various drugs [15]. More recently, Manciu et al. characterized the nucleotide-induced P-glycoprotein conformational changes in the presence of several anthracycline derivatives using acrylamide quenching [19].

The purpose of this work was to study the mode of interaction of nucleotide and three benzodiazepines (bromazepam, chlordiazepoxide and flurazepam) with catalytically active P-glycoprotein. For these purposes steady-state fluorescence was applied to characterize the environment of the P-glycoprotein Trp residues and the changes that take place upon drug or nucleotide binding. All benzodiazepines studied interact with the P-glycoprotein in the presence and absence of the nucleotide ATP and all the nucleotide affinity experiments revealed that the presence of benzodiazepines does alter the ATP affinity/conformation for P-glycoprotein NB domains. These results indicates the presence of different binding sites on the multidrug transporter for benzodiazepines and nucleotide and that there is conformational communication between the drug binding region of P-glycoprotein and the nucleotide catalytic site within the NB domains.

2. Materials and methods

DMPC was obtained from Avanti Polar Lipids. Imidazole, with low intrinsic fluorescence, and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid (CHAPS) were from Sigma. Ni^{2+}-nitrilotriacetic acid agarose (Ni-NTA) resin was from QIAGEN Inc. Bromazepam, chlordiazepoxide and flurazepam were a gift by Roche Diagnostic. All other chemicals were reagent grade from Sigma.

2.1. Pichia pastoris cells and microsome preparation

Metanotrophic yeast *P. pastoris* expressing mouse P-glycoprotein (Pgp) with a series of six histidines (MDR3-His_6) was grown as previously described [20,21]. Plasma membrane vesicles or microsomes were isolated from *P. pastoris* cells combining two methods of cell disruption, freeze–thaw lysis and glass bead vortexing. Frozen cells were rapidly thawed at 30 °C and then acid-washed glass beads (Sigma, 425–600 μm) were added, this mixture was vortexed (on the highest set) ten times for 1 min, each time keeping the cells on ice for 2–3 min between vortexing. The glass beads were recovered after centrifugation at 2500×*g* for 5 min, and subsequently washed in concentrated HCl, followed by extensive rinsing and drying for further use. The homogenate was then centrifuged at 14,000×*g* for 30 min to remove unbroken cells, nuclei and mitochondrial fractions. The supernatant was centrifuged at 150,000×*g* for 90 min and microsomes present in the pellet were resuspended in 50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM β-mercaptoethanol, 10 mM imidazole and protease inhibitors (pepstatin A and leupeptin at 10 μg/ml, and 1 mM of PMSF) at 2–4 mg/ml of total microsomal protein. Homogenization was achieved by the successive passages through 19- to 25-gauge needles, followed by concentration with a second centrifugation at 150,000×*g* for 60 min. Microsomes could be stored frozen at −20 °C, for 2 months.

2.2. Solubilization and purification of P-glycoprotein

Microsomes were thawed and solubilized in 26.5 mM CHAPS, 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM β-mercaptoethanol, 10 mM imidazole, and protease inhibitors (as above) at a protein final concentration of 2–4 mg/ml, for 30 min at 4 °C, with constant mixing by slowly rotation. The insoluble material was removed by centrifugation at 100,000×*g*, for 45 min. The conditions for purification on Ni^{2+}-NTA resin of the Pgp present on the soluble microsomal proteins were as described elsewhere [21].

2.3. Measurement of P-glycoprotein ATPase activity

Mg^{2+}-ATPase activity of Pgp was determined by measuring the release of inorganic phosphate from ATP, using a colorimetric method adapted from Chifflet et al. [22]. Pgp preparations (1.0–1.5 μg of protein) were diluted with assay buffer (50 mM Tris–HCl, 150 mM NH_4Cl, 5 mM $MgCl_2$, 1 mM EGTA/ouabain/sodium azide and 2 mM dithioerythritol, pH 7.4) to a final volume of 90 μl. The 96-well microtitre plate was incubated for 5 min at 37 °C. The reaction was initiated with the addition of 10 μl of ATP solution in assay buffer, giving a final concentration of 3 mM. The reaction was stopped after 20 min at 37 °C, with 90 μl of 6% SDS, 0.5% ammonium molybdate in 0.5 M HCl. For the reduction of the complex 10 μl (freshly prepared) of 30% ascorbic acid was added. The reaction products were stabilized with 100 μl of 2% sodium citrate, 2% sodium arsenite and 2% acetic acid. After 30 min in the dark, the absorbance at 750 nm was measured on a microplate spectrophotometer. Free hydrolysis of ATP in the colorimetric assay was subtracted. Potassium hydrogenophosphate was used as standard. Verapamil, a known Pgp ATPase stimulator, was used at two concentrations (10 and 15 μM).

2.4. Steady-state fluorescence experiments

Steady-state fluorescence measurements were carried out on a Perkin-Elmer LS-50 fluorescence spectrometer containing a constant temperature cell holder. In all measurements Trp excitation wavelength was 295 nm and emission data were recorded at 340 nm and temperature was kept at 37.0±0.1 °C. Emission spectra between 300 and 450 nm were collected after Trp excitation at 295 nm. The absorption of the samples was <0.1 at the excitation wavelength. All fluorescence experiments were conducted with 50 μg/ml Pgp in detergent-lipid buffer (2 mM CHAPS and DMPC at 0.72 mM in 50 mM Tris–Cl, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, pH 7.4). Previously liposomes were prepared by dryness with a stream of argon, of a DMPC solution in chloroform, followed by a 3 h film evaporation under vacuum. To the lipidic film 50 mM Tris–Cl buffer (pH 7.4) was added and this mixture was vortexed and extruded 10 times through polycarbonate filters (100 nm) to produce unilamellar liposomes. Stock solution of 100 mM ATP prepared in detergent-lipid buffer containing 2 mM CHAPS and 0.72 mM DMPC was added as 10 μl aliquots to 1 ml of 50 μg/ml Pgp, until a final ATP concentration of 6 mM. Benzodiazepine quenching experiments were carried out by successively adding 10 μl aliquots of drug working solutions, prepared in detergent-lipid buffer containing 2 mM CHAPS buffer and 0.72 mM DMPC, to 1 ml of Pgp solution in a 1 cm quartz cuvette, in the concentration range of 0–150, 0–80 and 0–190 μM for bromazepam, chlordiazepoxide and flurazepam, respectively. All fluorescence intensities were corrected for dilution and scattering. The effect of ATP and benzodiazepines absorption at the excitation wavelength (295 nm) was corrected according to Eq. (1) [23]:

$$I_{corr} = I_{exp}(A_T/A_{Pgp})((1-10^{-A}_{Pgp})/(1-10^{-A}_{T})) \quad (1)$$

where I_{corr} and I_{exp} correspond to the intensity of fluorescence corrected and experimentally obtained, respectively, and A_T and A_{Pgp} are the absorbances for the total solution and Pgp, respectively.

2.5. Routine procedures

The protein content of plasma membrane microsomes and Pgp preparations was determined by the microprotein Bradford assay [24], using bovine serum albumin (BSA) as standard. Protein samples were separated on 8% SDS-polyacrylamide gels, with no boiling prior to electrophoresis and were stained with silver.

2.5.1. Mathematical treatments

Based on the simplest binding site model, if a small-molecule ligand interacts with a bio-macromolecule and forms a static complex, the following equilibrium can be reached:

$$F + Q \Leftrightarrow FQ$$

where F is the fluorophore, Q is the quencher and FQ is the nonfluorescent complexes between both, and this type of static fluorescence can be described by the association constant, K_{FQ}:

$$K_{FQ} = [FQ]/([F][Q]) \quad (2)$$

This system can be interpreted in terms of the equation

$$F_0/F = 1 + K_{FQ}[Q] \quad (3)$$

where F_0 is the fluorescence intensity of the fluorophore and F is the fluorescence intensity of the fluorophore in the presence of the quencher.

When the plot of the above equation has an upward curvature, concave toward the y-axis, the system can be treated in terms of the sphere-of-action model, for static fluorescence [25]:

$$F_0/F = (1 + K_{FQ}[Q])\exp([Q]VN_A) \quad (4)$$

where V is the volume of the sphere and N_A is Avogadro's constant, and if $K_{FQ}[Q]$ is small enough, $(1+K_{FQ}[Q]) \approx \exp(K_{FQ}[Q])$, which is equivalent to $\exp([Q]VN_A)$ [26] Eq. (3) becomes:

$$F_0/F = \exp(K_{FQ}[Q]) \quad (5)$$

and K_{FQ} can be easily determined.

The system can also be interpreted in terms of the quenching data for ligands presenting a monophasic behavior by the equation describing a single type of binding site:

$$(\Delta F/F_0 \times 100 = \{(\Delta F_{max}/F_0 \times 100)[Q]\}(K_d + [Q]) \quad (6)$$

where $(\Delta F/F_0 \times 100)$ is the percentage of change in fluorescence relative to the initial value after addition of substrate at a concentration [Q], and K_d is the dissociation constant; an equation that is rather used to determine binding parameters for protein/ligand interaction as it is a simple linearization of a binding isotherm for fluorescence data [27–29].

The values of the binding constants were also refined by nonlinear least-squares regression with the program Hyperquad [30], for which it was possible to minimize simultaneously data for several wavelengths.

For the systems where two different quenchers were used (drugs and ATP), the model in Hyperquad was:

$$F + Q \Leftrightarrow FQ \quad K_{FQ} = |FQ|/|F| \cdot |Q|$$

$$F + Q_1 \Leftrightarrow FQ_1 \quad K_{FQ1} = |FQ_1|/|F| \cdot |Q_1$$

$$F + Q + Q_1 \Leftrightarrow FQQ_1 \quad K_{FQQ1} = |FQQ_1|/|F| \cdot |Q| \cdot |Q_1|$$

where F is the fluorophore, P-glycoprotein, Q is the drug, Q_1 is ATP and K_{FQ}, K_{FQ1}, K_{FQQ1} are the, respective, association constants.

3. Results and discussion

3.1. Solubilization and purification of P-glycoprotein

In earlier experiments to purify Pgp from *P. pastoris* plasma membrane, microsome proteins were extracted with the

zwitterionic detergent CHAPS, which required a high detergent/protein ratio (30:1, w/w). Detergent concentration, in the presence of 50 mM NaCl, for maximal Pgp solubilization was determined as 26.5 mM of CHAPS and the transporter remained soluble at 2 mM of the same detergent. Further purification of detergent-soluble supernatants was achieved by Ni-NTA affinity chromatography. Fig. 1 shows a silver-stained SDS-PAGE electrophoresis representing the overall of the Pgp preparation, with the final pure product in lane 4. An efficient Pgp solubilization and purification was achieved as determined by densitometric scans of the gel revealing an approximately 90% purity. The yield of CHAPS-purified Pgp from plasma membrane microsomes was about 200 μg of Pgp/mg of microsomal proteins.

3.2. Characterization of the ATPase activity of purified P-glycoprotein

To further characterize MDR3 Pgp Mg^{2+}-ATPase activity, ATP hydrolysis was measured in the final detergent preparation. Pgp-native (0.37 ± 0.02 μmol/min mg) and verapamil-stimulated (0.52 ± 0.04 μmol/min mg) ATPase activity were readily detectable after purification. Maximal stimulation was observed at 15 μM verapamil, which gave a 1.6-fold enhancement. The presence of inhibitors of the mitochondrial F1-ATPase (sodium azide), Na^+/K^+ (ouabain) and Ca^{2+}-ATPase (EGTA) did not affect the purified Pgp ATPase. This Pgp purified from *P. pastoris*, using CHAPS detergent, is fully functional and available for biophysical studies.

These results show that CHAPS solubilization yields pure Pgp with good levels of specific ATPase activity, which was characterized on the pure protein, free of added lipids. The highest levels of native and verapamil-stimulated ATPase activity for Pgp soluble in CHAPS were reported here. In our native protein preparation ATPase activity is 6-fold higher than DTT activated pure *P. pastoris* MDR1 Pgp isolated by DM solubilization and affinity chromatography [31] and 2-fold higher than that reported by Lerner-Marmarosh et al. [21] for DTT activated pure *P. pastoris* MDR3 P-glycoprotein solubilized by DM and purified by affinity chromatography and DEAE-cellulose chromatography. In addition, the overall yield of Pgp from the yeast culture approaches 200 μg of pure protein per g wet wt cells compared to 50 μg per g wet wt cells for the Lerner-Marmarosh et al. [21] preparation. Finally the entire microsome solubilization and purification procedure is convenient and completed within 3–4 h.

3.3. The effect of benzodiazepines and ATP on the intrinsic Trp fluorescence

In our CHAPS-Pgp purified preparation, Trp fluorescence was observed when Pgp in detergent-lipid buffer (2 mM CHAPS buffer and 0.72 mM DMPC) was excited at 295 nm and fluorescence emission was detected around 340 nm. The transporter is studied in its soluble form with the presence of phospholipids that has been found to greatly improve the extent and reproducibility of quenching by drugs and nucleotides [28]. Addition of ATP to Pgp resulted in a large quenching of the fluorescence (Fig. 2b), suggesting interaction of ATP to Pgp, as expected [10]. Pgp in the presence of all benzodiazepines (bromazepam, chlordiazepoxide and flurazepam) also show a large quenching but with a shift on the λ_{max} of Trp residues fluorescence emission. A small 4-nm red shift on emission maximum was observed with chlordiazepoxide, while 7- and 10-nm red shift were found for flurazepam and bromazepam, respectively. These results suggest the formation of an association between the macromolecule and the drugs with a change of Trp environment [15]. Furthermore, it is possible to state that the Pgp Trp residues emit fluorescence at 340 nm, which indicates that the Trp residues are in a relatively nonpolar environment [10], buried within the protein structure and, that not all Trp residues responsive for detectable fluorescence emission are located within the TM regions. As Trp residues are quite sensitive to the surrounding medium, changes in accessibility to solvent can be used to detect conformational changes upon binding of compounds to the protein and in the presence of all three benzodiazepines an association/conformational change on Pgp seems to occur, detected by the red shift on the maximum wavelength on the emission spectrum, and showing a change, of the Trp, to a more polar environment [15].

3.4. Quenching of Trp residues by benzodiazepines

To investigate the Pgp/drug interactions in more detail, fluorescence quenching titrations were carried out with increasing concentrations of each benzodiazepine (bromazepam, chlordiazepoxide and flurazepam), in the absence and presence of ATP. The quenching plots obtained for all drugs tested were nonlinear with an upward curvature (Fig. 3). This type of curvature can be a consequence of both static and dynamic effects in quenching [25] and to preclude the existence of dynamic quenching studies at different temperatures were performed (data not shown). As a rise in quenching with decrease in temperature was observed the existence of dynamic quenching was discarded. As demonstrated by Castanho and Prieto [26] pure static quenching by

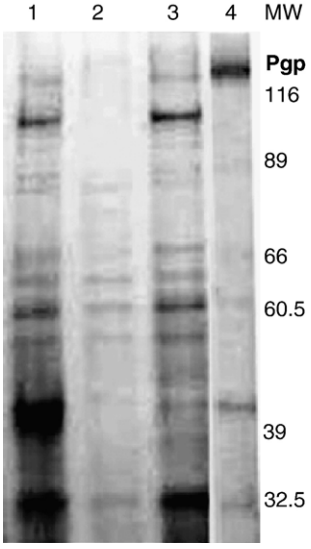

Fig. 1. Isolation and purification of mouse MDR3 Pgp. Silver staining of 8% SDS-PAGE gel containing 1.5 μg of CHAPS solubilized preparations. Lane 1: microsomes; lane 2: insoluble pellet after CHAPS microsomes solubilization; lane 3: soluble supernatant after CHAPS microsome solubilization; and lane 4: eluate from Ni^{2+}-NTA with 200 mM imidazole.

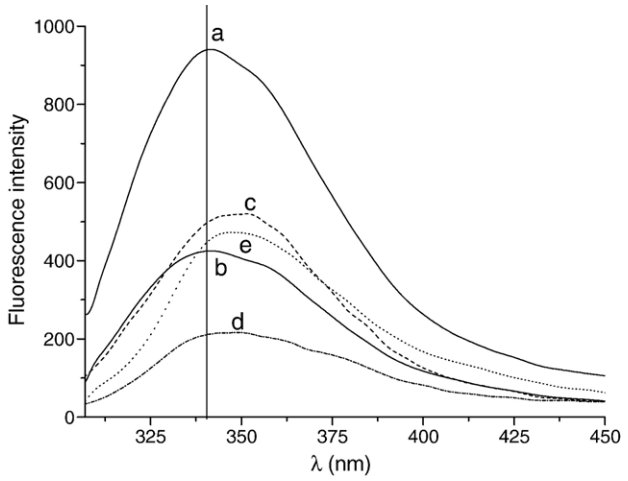

Fig. 2. Fluorescence emission spectra of P-glycoprotein Trp residues in the absence (a) and presence of 3 mM ATP (b), 240 μM bromazepam (c), 124 μM chlordiazepoxide (d), and 220 μM flurazepam (e). The concentrations used were close to saturation.

complexation can also lead to upward curvatures in Stern-Volmer plots (see Materials and methods) and considering that Trp residue quenching by benzodiazepines was due to a true static mechanism, we calculated the association quenching constant, K_{FQ}, using Eq. (5) which accounts for the association between the fluorophore and the quencher in the ground state. The association constants obtained for each benzodiazepine were further refined using the program Hyperquad (Table 1).

Following the same considerations the association constants of benzodiazepines with Pgp in the presence of ATP were also determined by Eq. (5) and further refined with program Hyperquad using the model suggested when two quenchers coexists (Table 1).

It is important to note that to compare the values obtained in this case Eq. (5) corresponds to an association constant described by the chemical equation

$$FQ_1 + Q \Leftrightarrow FQQ_1 \quad K^{FQ1}_{FQQ1} = |FQQ_1|/|FQ_1| \cdot |Q|$$

and that the value of K_{FQQ1} obtained by Hyperquad can be converted into this association constant using the values obtained for the association constant K_{FQ1} as $\log(K^{FQ1}_{FQQ1}) = \log(K_{FQQ1}) - \log(K_{FQ1})$. The values obtained are described in Table 1.

The values obtained for the association constants, K_{FQ}, confirm the strong association that is predicted when the quenchers and fluorophores actually form a ground-state complexation by a static quenching mechanism [25] and show that previous binding of the nucleotide to Pgp is not necessary for benzodiazepines interaction with the transporter. The values obtained by Eq. (5) and Hyperquad are identical which confirms the monophasic behavior of these drugs.

When Pgp bound to ATP is quenched by the same benzodiazepines the association constant values determined by Eq. (5) and Hyperquad are similar. Comparing the values of the association constants obtained, in the absence and presence of ATP, it is possible to conclude that the degree of affinity in the presence of ATP depends on the drug, a result also obtained by other authors [15,28].

3.5. Quenching of Trp residues by nucleotide

Titration of Pgp with increasing concentrations of ATP led to a saturable, concentration-dependent quenching with a hyperbolic tendency as expected (Fig. 4) [27,28]. Assuming an interaction with a single type of binding site, quenching data were used to

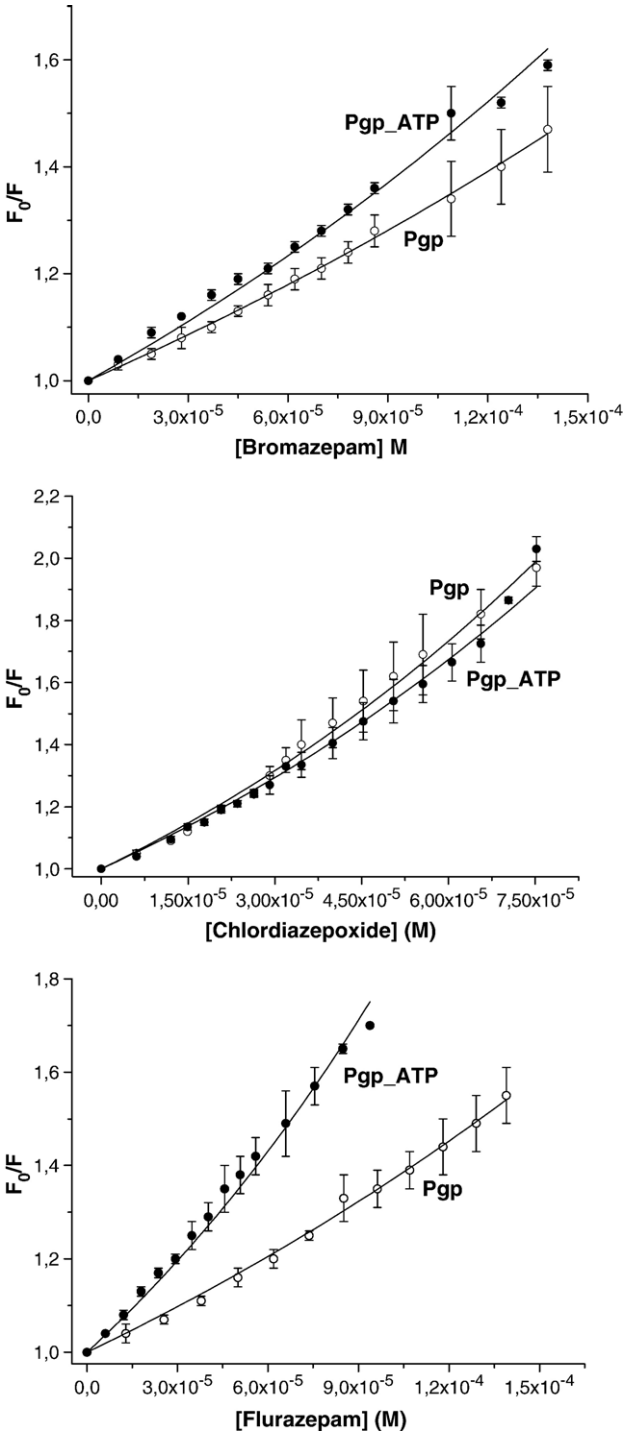

Fig. 3. Trp residue quenching of fluorescence by benzodiazepines in the absence (○) and presence of nucleotide (●). To 50 μg/ml Pgp in detergent-lipid buffer were added increasing concentrations of various benzodiazepines, and fluorescence at 340 nm was collected. The continuous line represents the best computer fit of the quenching data to a nonlinear Stern-Volmer plot using Eq. (5).

Table 1
Values of the association constant for benzodiazepines to Trp residues on Pgp in the absence and presence of nucleotide

	P-glycoprotein		
	$\log K_{FQ(Eq.\ (5))}$	$\log K_{FQ(Hyper)}$	
Bromazepam	3.42±0.05	3.30±0.05	
Chlordiazepoxide	3.93±0.02	3.92±0.03	
Flurazepam	3.52±0.09	3.53±0.03	
	P-glycoprotein+ATP		
	$\log K^{FQ1}_{FQQ1(Eq.\ (5))}$	$\log K_{FQQ1(Hyper)}$	$\log K^{FQ1}_{FQQ1(Hyper)}$
Bromazepam	3.54±0.06	6.40±0.13	3.71±0.13
Chlordiazepoxide	3.94±0.04	6.79±0.13	4.10±0.13
Flurazepam	3.80±0.06	6.80±0.14	4.11±0.08

Values for association constant are the means from at least three independent experiments, using different batches of protein.

estimate the parameters of nucleotide binding using Eq. (6) and further refining with the program Hyperquad (Table 2). Subsequently, experiments were taken to investigate whether the quenching of Trp residues with ATP in the presence of the benzodiazepines was independent and/or additive. So, each benzodiazepine was added to the transporter, and then Pgp bound to drug in detergent-lipid solution was titrated with increasing concentrations of ATP. Experimental data presented, once again, a saturable and concentration-dependent quenching for each drug (Fig. 4). In this case to compare the constants obtained by the two mathematical approaches a procedure similar to that describe in the previous section was used, Eq. (6) corresponds to an association constant described by the chemical equation

$$FQ + Q_1 \Leftrightarrow FQQ_1 \quad K^{FQ}_{FQQ1} = |FQQ_1|/|FQ| \cdot |Q_1|$$

and that the value of K_{FQQ1} obtained by Hyperquad can be converted into this association constant using the values obtained

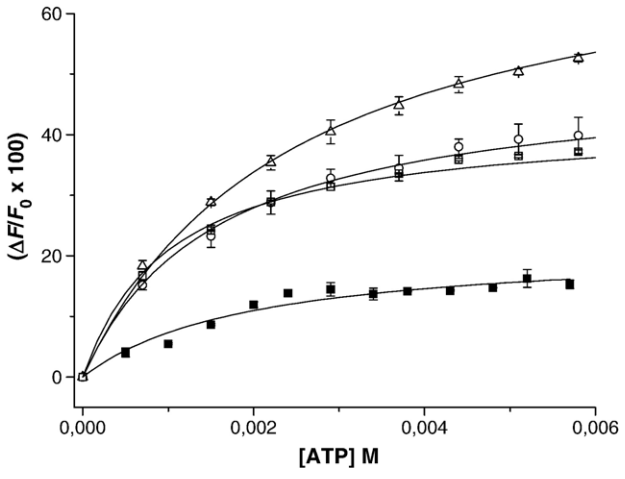

Fig. 4. Trp residue quenching of the fluorescence of Pgp (■) and Pgp containing benzodiazepines by addition of ATP. Each drug was added to 50 μg/ml Pgp at its saturating concentration: 238 μM bromazepam (○), 124 μM chlordiazepoxide (△), and 220 μM flurazepam (□). Fluorescence intensities at 340 nm were recorded following increasing concentrations of ATP. The continuous line represents the best computer fit of the quenching data to an equation describing one single type of binding site.

Table 2
The influence of ligand on the association constant determined for quenching of Pgp Trp by nucleotide in the absence and presence of drugs

	$\log K^{FQ}_{FQQ1(Eq.\ (6))}$	$\log K_{FQQ1(Hyper)}$	$\log K^{FQ}_{FQQ1(Hyper)}$
Pgp	2.69±0.03		2.66±0.10
Pgp_Bromazepam	2.77±0.04	6.65±0.02	3.30±0.08
Pgp_Chlordiazepoxide	2.60±0.03	6.52±0.06	2.60±0.06
Pgp_Flurazepam	2.99±0.01	6.99±0.06	3.46±0.06

Experimental quenching data shown in Fig. 4 were computer fitted to Eq. (6) using a nonlinear regression. Values are the means from at least two independent experiments. Each drug was added to 50 μg/ml Pgp close to its saturating concentration: 238 μM bromazepam, 124 μM chlordiazepoxide, and 220 μM flurazepam.

for the association constant K_{FQ} as $\log(K^{FQ}_{FQQ1}) = \log(K_{FQQ1}) - \log(K_{FQ})$. The estimated parameters of drug binding, by Eq. (6) and Hyperquad, are shown in Table 2.

The values obtained for the association constant of Pgp/ATP, by Eq. (6) and Hyperquad, are in the same range of those already described in literature. The association constant of several ABC superfamily of membrane transporters and ATP has already been determined and the values reported (determined for different pH and pMg concentrations) are mostly in the range of 2.46–3.40 unit log [15,28,32].

The values of the association constants of Pgp containing bound benzodiazepine to ATP determined by Eq. (6) and Hyperquad are slightly different but show the same trend. This result was expected as the use of Eq. (6) is a simplified model where it is assumed that all the proteins are bound to all benzodiazepines prior to the addition of ATP. Comparing the values of these association constants obtained by Hyperquad, it is possible to conclude that ATP binds to Pgp in the presence of bromazepam and flurazepam with a slightly different affinity degree as it had in their absence. Only the presence of chlordiazepoxide in the transporter seems to have no relevant change on the nucleotide apparent binding affinity, a result already observed for the other compounds that renders the transporter less sensitive to the allosteric actions of nucleotide [29]. In fact, a number of studies have shown that several compounds are able to elicit conformational changes in this ABC transporter [17,18,28,33,34] and the existence of direct communication between substrate binding and the ATP binding site has been already demonstrated [28]. Recently evidence that binding of transport substrate per se enhances ATP binding has been obtained for four ABC proteins [35–38]. These results appears in agreement with the proposal of the ATP-switch model by Higgins and Linton [39], where substrate binding to the transporter initiates the transport cycle by increasing the ATP binding affinity.

4. Concluding remarks

The purification method developed in this work, using detergent solubilization to extract Pgp from plasma membrane vesicles, gives an overall yield of P-glycoprotein from the yeast culture four times higher than that obtained by Lerner-Marmarosh et al. [21] and can be completed in 3–4 h. Its ATPase activity is also higher than that found for other methods [21,31] and is fully functional and available for biophysical studies.

The use of the intrinsic Trp fluorescence of Pgp allows to conclude that results obtained by fluorescence quenching studies show the existence of independent binding sites for benzodiazepines and ATP and that, as already observed for other families of drugs, the occupation of the drug and nucleotide binding site can occur in a random order [15,28], as shown by the use of a mathematical treatment that allows the determinations of simultaneous equilibria in solution and where the values of the overall stability constants ($\log K_{FQQ1(Hyper)}$) are identical, within experimental error. The slightly different values obtained by the two mathematical procedures could be expected since the use of Eqs. (5) and (6) is a simplified model where it is assumed that all the proteins are bound to all ATP/drug previous to the addition of the drug/ATP, which normally is not the case when there are simultaneous equilibria in solution.

Analysis of the λ_{max} shifts of the Trp residue fluorescence emission clearly proves that benzodiazepines, in the absence or presence of ATP, change the Trp environment and consequently the protein conformation. This fact shows that the association constants for the ternary systems Pgp/Benzodiazepines/ATP are apparent constants where the conformational changes cannot be discriminated. This is a situation commonly found in biochemical literature and the comparison of values determined by different groups must be carefully performed. Nevertheless it is possible to state that chlordiazepoxide does not change significantly the ATP association (binding or conformation), but bromazepam and flurazepam manipulate this association, maybe by a direct communication between drug binding and the ATP binding site. These results may be attributed to the different concentration of drugs into the membrane, showing that the affinity for Pgp can be related to the drug partition coefficients [40].

As a final conclusion, the results obtained for the interactions Pgp/Benzodiazepines/ATP support the existence of a precise requirement for benzodiazepines to alter ATP binding which appears to support the ATP-switch model [39–41] for Pgp transport mechanism, with the transport cycle being initiated by drug binding transmitting a conformational change to NBDs and controlling the binding of ATP.

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