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ORIGINAL ARTICLES

Benzodiazepine-Mediated Structural Changes in the Multidrug Transporter P-Glycoprotein: An Intrinsic Fluorescence Quenching Analysis

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Abstract P-glycoprotein expressed in *Pichia pastoris* was used to study the drug binding sites of different benzodiazepines. The effect of bromazepam, chlordiazepoxide, diazepam and flurazepam on P-glycoprotein structure was investigated by measuring the intrinsic fluorescence of the transporter tryptophan residues. Purified mouse mdr1a transporter in mixed micelles of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid and 1,2-dimiristoyl-*sn*-glycerol-3-phosphocholine emitted fluorescence at 340 nm indicative of the fluorophores in a relatively apolar environment. Acrylamide and iodide ion were used as collisional quenchers toward distinct regions of the transporter, the protein and the interface protein-surface, respectively. Binding of ATP induced conformational changes at the protein surface level in accordance with the location of the nucleotide binding sites. Bromazepam interaction with the transporter was located at the protein-surface interface, diazepam at the membrane region and chlordiazepoxide at the protein surface. Only the flurazepam interaction site was not detected by the quenchers used. All benzodiazepines were able to elicit reorientation of the protein fluorophores on the P-glycoprotein—ATP complex.

Keywords P-glycoprotein · Intrinsic fluorescence · Benzodiazepine · ATP · Acrylamide quenching · Iodide ion quenching

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Introduction

P-glycoprotein is a multidrug transporter capable of translocating numerous chemically and functionally unrelated compounds. It has been related to the presence of several drug binding sites responsible for this wide selectivity (Martin et al. 2000b; Shapiro et al. 1999; Shapiro and Ling 1997), localized within its 12 transmembrane domains (Bruggemann et al. 1989; Greenberger et al. 1991). This active transporter belongs to the ATP binding cassette superfamily (Germann 1996) and uses the energy from ATP hydrolysis produced at the two cytosolic nucleotide binding domains (al-Shawi and Senior 1993) to exert its transport function (Urbatsch et al. 1995). P-glycoprotein's mechanism of action requires both (1) ATP/drug binding and (2) binding site affinity changes/reorientation (Martin et al. 2001). Based on these actions responsible for the P-glycoprotein transport cycle, models were developed describing the sequence of binding site changes determined by nucleotide binding and hydrolysis (Higgins and Linton 2004; Sauna and Ambudkar 2000; Urbatsch et al. 1994). Urbatsch et al. (1994) and Sharom et al. (Sharom 1995; Sharom et al. 1995) have observed ATP hydrolysis stimulation after drug binding to the transmembrane domains. Evidence of communication between drug binding and nucleotide binding domains

comes from diverse authors (al-Shawi et al. 2003; Martin et al. 2000b, 2001; Mechetner et al. 1997; Shapiro and Ling 1997; Smith et al. 2000; Sonveaux et al. 1999) who used different biophysical techniques to demonstrate that the multidrug transporter undergoes tertiary structural changes in response to nucleotide binding and subsequent hydrolysis (Liu and Sharom 1996; Rosenberg et al. 2001, 2003; Sonveaux et al. 1996).

The mechanism of action for P-glycoprotein is, however, poorly understood and is fundamental to determine how the transporter recognizes so many structurally diverse compounds. Identification of the binding sites for substrates and inhibitors on P-glycoprotein has been the focus of many investigations. In the last decade, various experimental data were collected, but their contribution to clarifying the molecular basis of drug transport and function of P-glycoprotein was small. Presently, the number and location of the drug binding sites remain unclear. After the initial idea of a large common binding site (Borgnia et al. 1996) was abolished, a minimum of two binding sites dependent on the cooperative interaction between P-glycoprotein modulators had some support. Shapiro and coworkers postulated two distinct binding sites for rhodamine and Hoechst 33342, which interacted in a positively cooperative manner, and a third regulatory binding site for progesterone and prazosin (Shapiro et al. 1999; Shapiro and Ling 1997). From radioligand binding experiments, at least four different binding sites were inferred that were able to allosterically communicate (Martin et al. 2000a). Drug photolabeling studies performed by Safa (2004) described seven binding sites that partially interacted with each other. Loo and Clarke (2001a, b, 2002) conducted several cross-linking experiments on the interaction and transport of P-glycoprotein and proposed that the transmembrane domains TM2/TM11 and TM5/TM8 must enclose the drug binding pocket at the cytoplasmic site of P-glycoprotein. These authors also anticipated that P-glycoprotein binds substrates through an induced-fit mechanism, where the size and shape of the substrate change the packing of the transmembrane segments (Loo et al. 2003; Loo and Clarke 2002, 2005). Substrate-induced conformational changes in P-glycoprotein have been described for several compounds, such as progesterone and cyclosporin A (Loo et al. 2003), anthracycline derivatives (Sonveaux et al. 1999) and vinblastine or verapamil (Wang et al. 1998) using distinct biophysical methods. Recently, sensitivity of P-glycoprotein tryptophan residues to binding of benzodiazepines and ATP was described using the intrinsic fluorescence of the multidrug transporter (Lima et al. 2007). Here, we propose a substrate-induced conformational mechanism to understand how benzodiazepines interact with P-glycoprotein.

Benzodiazepines belong to a class of anxiolytic drugs that are among the most commonly prescribed drugs in the United States and Europe today. To have their therapeutic behavior, benzodiazepines need to reach the brain and, therefore, must cross the blood—brain barrier, which is composed of capillary endothelial cells expressing transporter proteins, responsible for the substrate efflux from the endothelial cells of the blood—brain barrier to the blood circulation. P-glycoprotein and the breast cancer resistance protein are two of the proteins involved in the efflux process at the blood–brain barrier.

The present investigation used an intrinsic fluorescence approach to elucidate the tertiary conformational changes of the multidrug transporter during the catalytic cycle by events in the transmembrane region resulting from benzodiazepine binding.

Materials and Methods

Materials

Bromazepam, chlordiazepoxide, diazepam and flurazepam (Fig. 1) were a gift from Roche Diagnostic (Indianapolis, IN). 1,2-Dimiristoyl-sn-glycerol-3-phosphocholine (DMPC) was obtained from Avanti Polar Lipids (Birmingham, AL). Imidazole, with low intrinsic fluorescence, and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid (CHAPS) were from Sigma-Aldrich (St. Louis, MO). Ni^{2+}-nitrilotriacetic acid agarose (Ni-NTA) resin was from Qiagen (Valencia City, CA). All other chemicals were reagent-grade from Sigma-Aldrich.

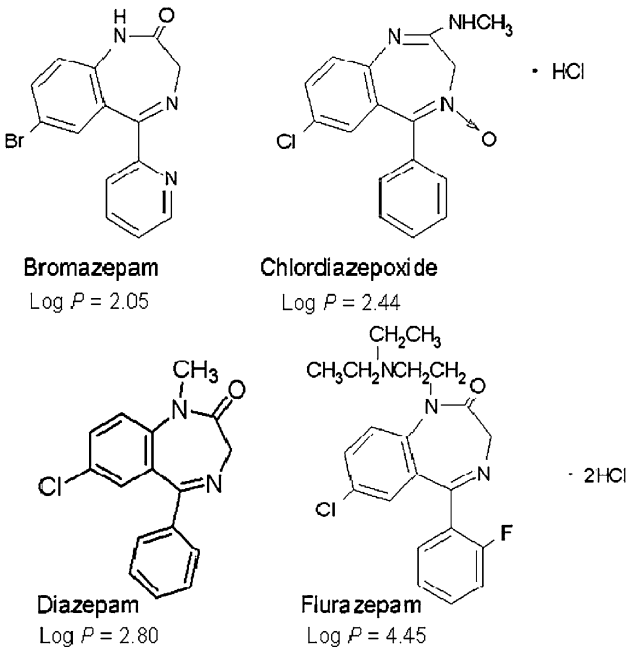

Fig. 1 Chemical structures of benzodiazepines investigated (log *P* represents the partition coefficient in water/octanol (Hansch 1989))

P-Glycoprotein Preparation

Metanotrophic yeast *Pichia pastoris* expressing mouse P-glycoprotein was grown as previously described (Beaudet et al. 1998; Lerner-Marmarosh et al. 1999; Lima et al. 2005). Plasma membrane vesicle preparation as well as CHAPS P-glycoprotein solubilization and purification were carried out according to previous work (Lima et al. 2007).

Steady-State Fluorescence Experiments

Steady-state fluorescence measurements were carried out on a Perkin-Elmer (Boston, MA) LS-50 fluorescence spectrometer containing a constant temperature cell holder at 37.0 ± 0.1°C and 10 x 10 mm quartz cuvette with constant stirring. Emission spectra between 300 and 450 nm were collected after tryptophan excitation at 295 nm, to ensure that the light was absorbed almost entirely by tryptophan residues and the absorption of the samples was < 0.1 at the excitation wavelength. In all measurements the fluorescence intensity was properly corrected for dilution, scattering and inner filter effect (Mateo et al. 2000).

Acrylamide and Iodide Quenching

Acrylamide and iodide stock solutions of 5 M were freshly prepared and added successively as 5-µl aliquots to the quartz cuvette containing 1 ml P-glycoprotein solution. To prevent I_3^- formation, 0.1 M $Na_2S_2O_3$ was added to the KI stock solution. In all experiments P-glycoprotein was kept in mixed micelles at ~30 µg/ml in a 2 mM CHAPS assay buffer (50 mM Tris-Cl, 50 mM NaCl, 5 mM MgCl2, 1mM β-mercaptoethanol, pH 7.4) in the presence of DMPC at 0.72 mM to ensure reproducibility (Sharom et al. 1995). Stock solution of 100 mM ATP was prepared in the same assay buffer and added to the P-glycoprotein solution at a final concentration of 3 mM. Quenching data were recorded at 340 nm after tryptophan excitation at 295 nm.

Routine Procedures

The protein content of plasma membrane vesicles and P-glycoprotein preparations was determined by the Bradford assay (Bradford 1976), using bovine serum albumin as standard.

Statistical Analysis

Statistical analysis for significant differences was performed using the two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

Mathematical Treatments

Linear quenching data were analyzed using the Stern-Volmer equation (Lakowicz 1999):

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

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively; [Q] is the concentration of quencher; and K_{sv} is the Stern-Volmer quenching constant.

When the quenching data, treated with equation 2, exhibited a downward curvature, toward the *x* axis, the data were analyzed using a modified Stern-Volmer equation (Lakowicz 1999):

$$F_0 - F = F_{0a} \bullet (K_{sva}\,[Q])/(1 + K_{sva}\,[Q]) \quad (2)$$

where F_{0a} is the fluorescence intensity in the absence of quencher of the accessible fraction and F is the fluorescence intensity in the presence of quencher, K_{sva} is the Stern-Volmer quenching constant of the accessible fraction and [Q] is the concentration of quencher.

Results and Discussion

Mouse Mdr1a P-glycoprotein expressed on *P. pastoris* yeast cells was purified and solubilized in a mixed micelle suspension containing CHAPS and DMPC (Lima et al. 2007; Sharom et al. 2001, 1995). The tryptophan fluorescence varies according to the surrounding environment's polarity and the presence of substrates (Lakowicz 1999; Sharom et al. 2005); therefore, it was necessary to characterize the intrinsic P-glycoprotein fluorescence in this mixed micelle suspension.

Characterization of P-Glycoprotein Intrinsic Fluorescence

To characterize the P-glycoprotein intrinsic fluorescence in mixed micelles, two collisional agents, acrylamide and iodide ion, were used. As ATP is a substrate involved in the transport function of this protein, the quenching experiments were carried out also in its presence. When selectively excited, the P-glycoprotein tryptophan residues emit fluorescence at 340 nm, which indicates that the fluorophores are in a relatively hydrophobic environment and that not all tryptophan residues responsive for detectable fluorescence emission are located within the protein membrane regions.

Collisional quenching with acrylamide was measured at 0–0.5 M, resulting in a linear Stern-Volmer plot, which suggests that all fluorophores accessible to the quenchers are in a similar environment (Fig. 2). Acrylamide

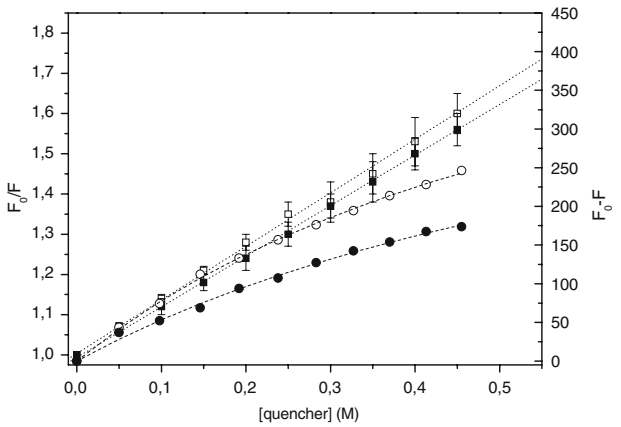

Fig. 2 Acrylamide and iodide ion quenching of tryptophan residue fluorescence. Quenching data of P-glycoprotein in the absence (□) and presence (■) of nucleotide by acrylamide were fitted to a linear Stern-Volmer plot (*dotted lines*) using equation 1. Quenching data of P-glycoprotein in the absence (○) and presence (•) of nucleotide by iodide ion were fitted to a nonlinear Stern-Volmer plot (*dashed lines*) using equation 2

quenching was also carried out in the presence of saturating concentration of ATP. Changes in tryptophan accessibility may occur upon substrate binding and can be detected using acrylamide quenching (Lakowicz 1999). As shown in Fig. 2, addition of ATP resulted only in a very small change (~10%) in the Stern-Volmer constant, indicating a slight decrease in fluorophore accessibility to solvent. These results confirm that no major changes take place on the tryptophan residues available for the action of acrylamide after nucleotide binding, as was previously reported by Sonveaux et al. (1999) and Liu et al. (Liu and Sharom 1997; Liu et al. 2000). The nucleotide binding domains in P-glycoprotein are located at the transporter surface out of the membrane region; therefore, acrylamide is able to quench the transporter's intrinsic fluorescence at the membrane level of P-glycoprotein in mixed micelles.

To gain more information on the protein structure, other quenchers can be used, such as the iodide ion quencher, which cannot enter the nonpolar region of proteins and, thus, specifically quenches surface fluorophores (Lakowicz 1999; Moro et al. 1993). Loss of fluorescence intensity of P-glycoprotein tryptophan residues with increasing concentrations of iodide ion, measured up to 0.5 M, resulted in a nonlinear Stern-Volmer plot with a downward curvature toward the *x* axis, indicating that there are two fluorophore populations present and one class of fluorophore is not quite accessible to the quencher (Fig. 2). Addition of nucleotide to P-glycoprotein resulted in a decrease (~25%) in the Stern-Volmer constant of the accessible fraction (K_{sva}) as well as in the initial fluorescence intensity, as represented in Fig. 2. Apparently, iodide ion detects ATP binding to P-glycoprotein, confirming that this quencher, under this experimental condition, acts at the level of nucleotide binding domains, i.e., at the protein surface.

P-Glycoprotein Conformational Changes Induced by Benzodiazepines

It is known that P-glycoprotein interacts with at least four benzodiazepines (bromazepam, chlordiazepoxide, diazepam and flurazepam) by conformational changes and/or binding (Lima et al. 2007). The binding constants were previously determined for each one of these drugs, but no structural information about the P-glycoprotein–benzodiazepine complex formed was described. Quenching of the intrinsic transporter fluorescence using collisional quenchers acrylamide and iodide ion allows characterization of the protein conformational state as it interacts with each benzodiazepine agent (Lakowicz 1999).

Acrylamide quenching of tryptophan fluorescence was carried out in the presence of saturating concentrations of four benzodiazepines (240 μM bromazepam, 125 μM chlordiazepoxide, 120 μM diazepam and 220 μM flurazepam), to detect any change in the exposure of fluorophores to the aqueous environment upon drug interaction.

The P-glycoprotein–benzodiazepine interaction resulted in a linear behavior, indicating the presence of one class of tryptophan accessible to the quencher, most probably buried in the protein membrane region. A significant increase in the slope of the Stern-Volmer plot is clear in Fig. 3 for the P-glycoprotein–bromazepam (45%) and P-glycoprotein–diazepam (42%) complexes, revealing that these drugs do not interact close to a tryptophan residue at the protein membrane region. These two benzodiazepines induce statistically significant changes (K_{sv} for bromazepam

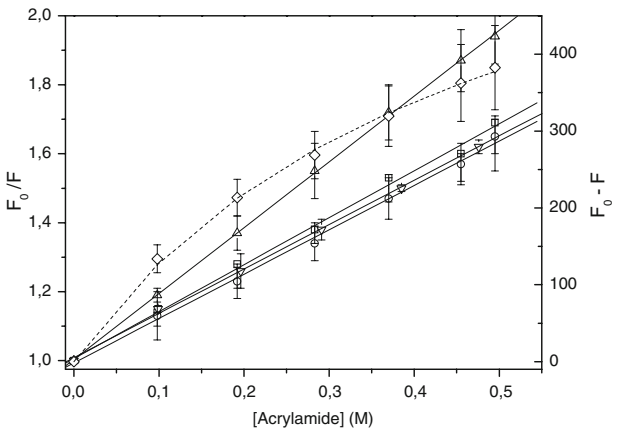

Fig. 3 Acrylamide quenching of P-glycoprotein tryptophan residue (□) fluorescence in the presence of bromazepam (Δ), chlordiazepoxide (◯), diazepam (◇) and flurazepam (▽). The results correspond to the mean ± standard deviation of at least three independent experiments

$1.85 \pm 0.10\ M^{-1}$, $P < 0.001$, and K_{sv} for diazepam $1.80 \pm 0.14\ M^{-1}$, $P < 0.001$ vs. P-glycoprotein alone $1.27 \pm 0.03\ M^{-1}$) on the multidrug transporter membrane region. Bromazepam/diazepam association induces reorientation of these fluorophores toward the protein surface, as confirmed by the red shift on the fluorescence emission wavelength from 340–350 and 343 nm, respectively (data not shown). At saturating concentrations chlordiazepoxide and flurazepam did not induce reorientation on the membrane fluorophores detectable by acrylamide quenching, as indicated by the K_{sv} values (1.19 ± 0.03 and $1.21 \pm 0.08\ M^{-1}$, respectively; $P < 0.05$ vs. P-glycoprotein).

Quenching assays were conducted for P-glycoprotein in the presence of saturating concentrations of all benzodiazepines with iodide ion up to 0.5 M. For each drug a nonlinear behavior was observed (not shown), which was analyzed according to Lakowicz (1999), allowing calculation of a Stern-Volmer constant value for the accessible fraction of fluorescence (K_{sva}), summarized in Table 1.

Analysis of the F_a values in Table 1 revealed that only 51% and 61% of the P-glycoprotein surface fluorophores were available for quenching by iodide ion after the interaction with bromazepam and chlordiazepoxide, respectively. These results are 10–20% lower than the tryptophan residues accessible for the quencher in the multidrug transporter that indicates a reorientation of the surface fluorophores toward the protein hydrophobic region as a consequence of benzodiazepine interaction. The K_{sva} values obtained in the presence of bromazepam and chlordiazepoxide are statistically different when compared with the transporter itself. Considerable conformational changes arise from these drugs' interaction with P-glycoprotein, resulting in a higher exposure of the surface tryptophan residues to the environment, as the 2.5- and 3.0-fold increases in the K_{sva} values for bromazepam and chlordiazepoxide, respectively, indicate (Table 1). These two benzodiazepines must interact far from fluorophores at the protein surface, and both are able to induce a reorientation of these to a polar environment, as suggested by the decrease in F_a values.

Table 1 The Stern-Volmer constant for the accessible fraction of fluorescence and the initial fraction of fluorescence intensity values obtained by the intrinsic P-glycoprotein fluorescence quenching using iodide ion

	K_{sva} (M^{-1})	F_a
P-glycoprotein	1.46 ± 0.08	0.70 ± 0.06
P-glycoprotein–bromazepam	3.61 ± 0.17**	0.51 ± 0.02*
P-glycoprotein–chlordiazepoxide	4.31 ± 0.30**	0.41 ± 0.10*
P-glycoprotein–diazepam	1.66 ± 0.02*	0.61 ± 0.23*
P-glycoprotein–flurazepam	1.50 ± 0.04	0.61 ± 0.07

Values for K_{sva} are the means ± error ($n = 3$) determined by equation 2 from at least three independent experiments, using different batches of protein. Benzodiazepines were present at saturating concentrations (240 μM bromazepam, 125 μM chlordiazepoxide, 120 μM diazepam and 220 μM flurazepam). * $P < 0.05$; ** $P < 0.001$ vs. P-glycoprotein in mixed micelles

P-glycoprotein undergoes small surface conformational changes in the presence of diazepam, as suggested by the 15% increase in the K_{sva} and the absence of variation in the F_a value, within experimental error. These results indicate that the P-glycoprotein–diazepam interaction must be away from the surface tryptophan residues.

Flurazepam is the only benzodiazepine that induces no detectable changes, by iodide quenching, on the surface P-glycoprotein fluorophores since both F_a and K_{sva} values remain identical to those obtained with the transporter.

The collisional quenching experiments demonstrated that benzodiazepine binding, except for flurazepam, induces a rearrangement on the transporter, which probably leads to the transport of drugs to the outside of the cell. Several unrelated compounds and even nucleotides have been reported to promote conformational changes in the ABC transporters during their mechanism of action (Higgins and Linton 2004; Manciu et al. 2001; Martin et al. 2000b; Smith et al. 2000). Manciu et al. (2001) revealed that daunorubicin and doxorubicin lead to conformational changes of multidrug-resistance protein 1 when this ABC transporter is catalytically active. Also, Sonveaux et al. (1999) reported tertiary structural changes in P-glycoprotein induced by anthracycline derivatives and ATP.

As acrylamide acts at the membrane region of the transporter far from the lipid–protein interface and iodide ion quenches preferentially fluorophores at the protein cytosolic surface, the benzodiazepine-induced conformational changes in P-glycoprotein can be localized on its structure. Taking together the collisional quenching data, it is possible to suggest a probable site for each P-glycoprotein–benzodiazepine interaction; and a schematic representation of these is represented in Fig. 4.

According to the collisional quenching studies performed with acrylamide and iodide ion on P-glycoprotein in the absence and presence of benzodiazepines, it is possible to establish the protein membrane–surface interface for the site of interaction for bromazepam, the surface region for chlordiazepoxide and the membrane region for diazepam. No conclusion can be drawn concerning the site of interaction for flurazepam on the mutlidrug transporter based on the results obtained.

Accordingly, with the 11 Trp position in P-glycoprotein (Liu et al. 2000), these results suggest that bromazepam must have a binding region that must affect the fluorescence of Trp44 in the N-terminal tail, Trp159 in the first

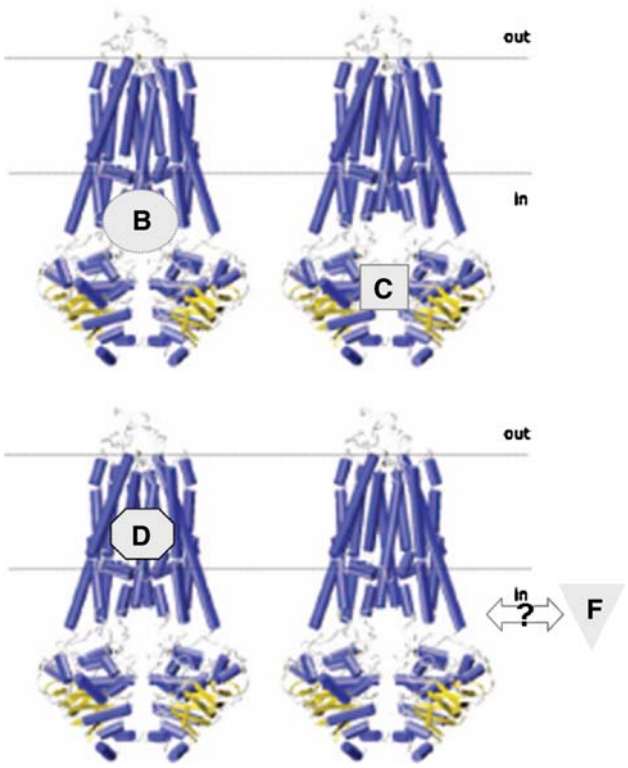

Fig. 4 Benzodiazepine interaction areas in a stereoimage of a P-glycoprotein homology model generated on the basis of the X-ray structure of Vc-MsbA (adapted by Globisch et al. 2008). *B* represents the putative bromazepam site of interaction; *C* represents the putative chlordiazepoxide site of interaction; and *D* represents the putative diazepam interaction site. For flurazepam (*F*) no putative site could be attributed

cytoplasmic loop and Trp209 in the second extracellular loop. Furthermore, the diazepam binding region must be close to Trp133, Trp229 and Trp312 in TM2, TM4 and TM5, respectively—all in the transmembrane region. For chlordiazepoxide, which binds on the surface region, the fluorescence changes must be due to Trp695 and Trp705 in the linker region immediately following nucleotide binding site 1, Trp800 in cytoplasmic loop 3 and Trp852 in extracellular loop 5.

Conformational Changes in the P-Glycoprotein–ATP Complex Induced by Benzodiazepines

The P-glycoprotein multidrug transporter binds ATP and turns to the catalytically active condition, ready to transport drugs across the cell membrane. Is this process associated with reorientation of nucleotide and drug binding sites? Some authors have described changes in the catalytically active transporter conformational state induced by drug binding (Loo et al. 2003; Mechetner et al. 1997; Smith et al. 2000). By collisional quenching of the intrinsic P-glycoprotein–ATP complex fluorescence in the presence of each benzodiazepine, it is possible to detect conformational change in the protein membrane and surface regions.

Acrylamide quenching data for the transporter in the presence of all benzodiazepines exhibited a linear behavior of the Stern-Volmer relation, while the anionic quencher displayed a downward curvature toward the x axis. The Stern-Volmer constants were determined for each quencher and are listed in Table 2.

When the P-glycoprotein–ATP complex interacts with bromazepam or diazepam, a 90% or 36% increase, respectively, in the Stern-Volmer constant induces a reorientation of the protein membrane fluorophores to its exterior. Conformational changes were also observed with the tryptophan residues present on the transporter surface as indicated by the 100% increase in the K_{sva} value for bromazepam and diazepam. Only 50% of the protein surface fluorophores are accessible for the action of iodide ion on the catalytic active transporter. The fraction of fluorophores accessible to the iodide ion quencher remains identical, within experimental error, whether or not these drugs are present in the P-glycoprotein–ATP complex.

The interaction of chlordiazepoxide or flurazepam with the catalytically active transporter leads to a decrease of 35% or 55%, respectively, in the K_{sv} value determined by the action of acrylamide. The Trp residues located at the protein membrane region become less accessible to this quencher, suggesting (1) a chlordiazepoxide/flurazepam binding site close to these fluorophores or (2) a

Table 2 Stern-Volmer constants (K_{sv} and K_{sva}) determined by acrylamide and iodide quenching, respectively, and the corresponding linear correlation coefficient and F_a obtained for the P-glycoprotein–ATP complex in the presence of benzodiazepines

	K_{sv} (M^{-1})	r^2	K_{sva} (M^{-1})	F_a
P-glycoprotein–ATP	1.22 ± 0.05	0.994	1.11 ± 0.10	0.54 ± 0.06
P-glycoprotein–ATP–bromazepam	2.33 ± 0.01	0.999	2.24 ± 0.05	0.60 ± 0.09
P-glycoprotein–ATP–chlordiazepoxide	0.78 ± 0.02	0.996	2.29 ± 0.21	0.40 ± 0.01
P-glycoprotein–ATP–diazepam	1.66 ± 0.07	0.998	2.24 ± 0.11	0.47 ± 0.02
P-glycoprotein–ATP–flurazepam	0.55 ± 0.02	0.995	1.89 ± 0.11	0.50 ± 0.03

Values for K_{sv} and K_{sva} are the means ± error ($n = 3$) determined by equations 1 and 2, respectively, using different batches of protein. Benzodiazepines were present at saturating concentrations (240 μM bromazepam, 125 μM chlordiazepoxide, 120 μM diazepam and 220 μM flurazepam) and ATP at 3 mM

reorientation of the tryptophan toward a more hydrophobic environment on the protein.

According to the Lehrer analysis (Lakowicz 1999) of the iodide ion quenching data, the fluorophores accessible to the quencher are identical to the complex P-glycoprotein–ATP and the tertiary complexes formed with the benzodiazepines. At the catalytically active transporter surface, significant changes were verified with the interaction of chlordiazepoxide and flurazepam as revealed by the increase in K_{sv} values (100% and 70%, respectively). Contrary to what was observed at the transporter membrane region, on its surface the interaction of these two benzodiazepines induces changes far from the fluorophores.

The results from the conformational changes induced by the four benzodiazepines on the catalytically active transporter distinguish two groups based on the protein regions affected by the drug binding. Bromazepam and diazepam interaction lead to reorientation of both membrane and surface fluorophores toward a hydrophilic environment, while chlordiazepoxide and flurazepam induce the membrane and surface fluorophores to distinct environments, more hydrophobic or hydrophilic, respectively. These differences may be related to the drug size, shape and physical properties (Seelig 1998).

According to the vacuum cleaner model proposed for drug recognition by P-glycoprotein, drug partition in the membrane phospholipids rules its passive diffusion into the cell for transporter identification and subsequent efflux to the outside of the cell (Raviv et al. 1990). Benzodiazepine drug partition in phospholipid vesicles is poorly studied; a value was found for chlordiazepoxide distribution within DMPC liposomal vesicles (Rodrigues et al. 2001a, b) which is quite different from the octanol/water partition coefficient (P) available in the literature. Nevertheless, the P values are indicative and suggest that all benzodiazepines are able to partition in a two-phase system; therefore, these drugs may be recognized and subsequently bind to P-glycoprotein through an induced-fit mechanism at least in two protein different regions: *membrane* for bromazepam and diazepam and *surface* for chlordiazepoxide and flurazepam.

Several authors through numerous biophysical techniques have proposed that P-glycoprotein exists in different conformations during the transport process from the drug and/or nucleotide binding to the ATP hydrolysis and drug release to the outside of the cells (Liu and Sharom 1996; Mechetner et al. 1997; Sonveaux et al. 1996). Based on the experiments reported here, a model is proposed to clarify the P-glycoprotein–benzodiazepine interaction mechanism of transport.

The multidrug transporter interacts with benzodiazepines or ATP corresponding to a conformational state (I or I', respectively, in Fig. 5). Conformational state I reproduces the changes that occur at the protein membrane and surface regions according to the drug present. The simultaneous binding of benzodiazepines and ATP leads to a conformational state (II and II'). Substrate binding stimulates ATPase activity on P-glycoprotein, which elicits ATP hydrolysis and leads to a conformational state (III). This reorientation together with the energy from the hydrolysis allows the translocation of the drug on the transporter and consequently its release. Identical conformational states (I, I', II and III) were described for P-glycoprotein in the presence of vinblastine, verapamil, colchicine and doxorubicin (Adriamycin) (Chambers et al. 1990; Drose et al. 1993; Martin et al. 2001).

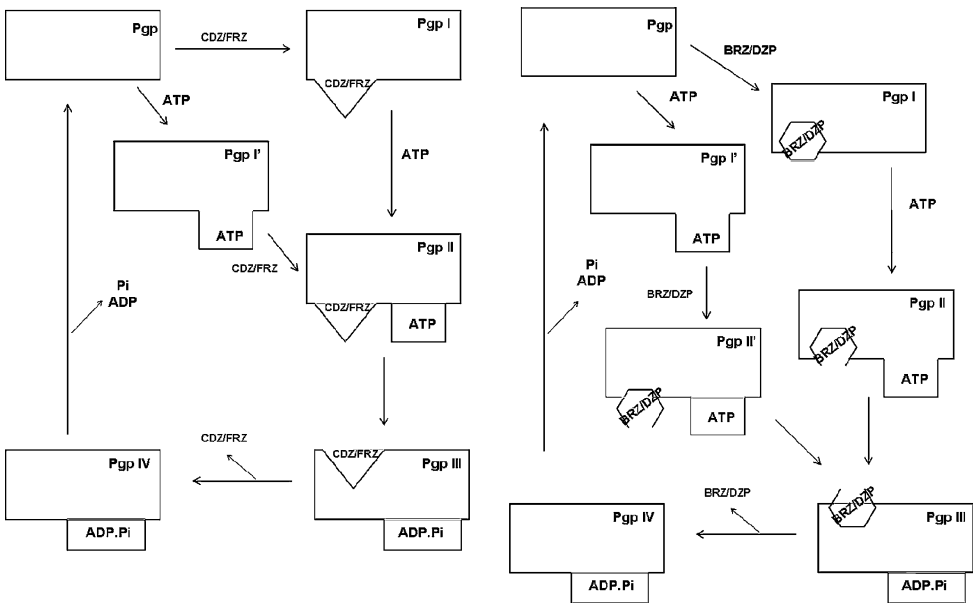

Fig. 5 Proposal for the mechanism of action for the P-glycoprotein–benzodiazepine interactions in the presence of ATP. The schematic representations correlate ATP binding/hydrolysis with the interaction of chlordiazepoxide (CDZ) and flurazepam (FZP) on the *left* and bromazepam (BRZ) and diazepam (DZP) on the *right*

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