Intermediate Structural States Involved in MRP1-mediated Drug Transport

ROLE OF GLUTATHIONE*

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Human multidrug resistance protein 1 (MRP1) is a member of the ATP-binding cassette transporter family and transports chemotherapeutic drugs as well as diverse organic anions such as leukotriene LTC4. The transport of chemotherapeutic drugs requires the presence of reduced GSH. By using hydrogen/deuterium exchange kinetics and limited trypsin digestion, the structural changes associated with each step of the drug transport process are analyzed. Purified MRP1 is reconstituted into lipid vesicles with an inside-out orientation, exposing its cytoplasmic region to the external medium. The resulting proteoliposomes have been shown previously to exhibit both ATP-dependent drug transport and drug-stimulated ATPase activity. Our results show that during GSH-dependent drug transport, MRP1 does not undergo secondary structure changes but only modifications in its accessibility toward the external environment. Drug binding induces a restructuring of MRP1 membrane-embedded domains that does not affect the cytosolic domains, including the nucleotide binding domains, responsible for ATP hydrolysis. This demonstrates that drug binding to MRP1 is not sufficient to propagate an allosteric signal between the membrane and the cytosolic domains. On the other hand, GSH binding induces a conformational change that affects the structural organization of the cytosolic domains and enhances ATP binding and/or hydrolysis suggesting that GSH-mediated conformational changes are required for the coupling between drug transport and ATP hydrolysis. Following ATP binding, the protein adopts a conformation characterized by a decreased stability and/or an increased accessibility toward the aqueous medium. No additional change in the accessibility toward the solvent and/or the stability of this specific conformational state and no change of the transmembrane helices orientation are observed upon ATP hydrolysis. Binding of a non-transported drug affects the dynamic changes occurring during ATP binding and hydrolysis and restricts the movement of the drug and its release.

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‡ The abbreviations used are: MRP1, multidrug resistance protein 1; Fgg, F-glycoprotein; NBD, nucleotide binding domain; TM, transmembrane segments; PCE, 3-[3-(oxornithinophenolino)xoxorubicin; DOX, doxorubicin; AMP-PNP, 5′-adenyl-β-γ-imidodiphosphate; ATR-FTIR, attenuated total reflection Fourier transform infrared; H/D, hydrogen/deuterium; ABC, ATP-binding cassette; BAPNA, Nα-benzoylarginine-

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proteoliposomes have been shown previously to exhibit both ATP-dependent drug transport and drug-stimulated ATPase activity (34).

We have utilized limited proteolytic cleavage and infrared spectroscopy to identify and characterize distinct intermediate structural states. The data suggest that GSH induces structural modifications that allow the coupling between drug transport and ATP hydrolysis. Binding of ATP, rather than hydrolysis, increases the accessibility of MRP1 to the aqueous medium. Partial conformational change may be required for the release of the drug in the external medium. No additional change in the accessibility toward the solvent and no change in the orientation of the transmembrane helices are observed upon ATP hydrolysis. Therefore we suggest that ATP hydrolysis could change the affinity of the substrate through conformational changes involving rotation of the transmembrane helices along their axis.

Binding of a non-transferred drug reorganizes the membrane-embedded domains in a conformation that restricts the movement of the drug and its release.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, AMP-PNP, GSH, doxorubicin (DOX), and N-α-benzoylarginine-t-p-nitroanilide (BAPNA) were purchased from Sigma. FCE (3′-3′-methoxyphosphorphino/doxorubicin) was supplied by Farmatia. 

**Purification and Reconstitution of MRP1**—Stable baby hamster kidney (BHK-21) cell line expressing MRP1 was generated by using procedures described previously (35). The conditions of expression and purification were as described (35). Reconstitution of the purified MRP1 was achieved using SM-2 Bio-Beads to remove detergent from protein/detergent/lipid mixture as described previously (34).

**Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR)**—ATR-FTIR spectra were recorded, at room temperature, on a Bruker IFS55 FTIR spectrophotometer equipped with a liquid nitrogen-cooled mercury-cadmium-telluride detector at a nominal resolution of 2 cm⁻¹ and encoded every 1 cm⁻¹. The spectrophotometer was continuously purged with air dried on a FTIR purge gas generator 75-62 Balston (Maidstone, UK) at a flow rate of 5.8 liters/min. The internal reflection element (ATR) was a germanium plate (50 × 20 × 2 mm) with an aperture angle of 45°, yielding 25 internal reflections (36).

**Sample Preparation**—The sample contained 20 μg of reconstituted MRP1. For the measurements carried out in the presence of different ligands, the molar protein to ligand ratio is 1:14 for DOX and FCE, 1:3 for GSH, and 1:6 for ATP or AMP-PNP. Thin films of oriented multilayers were obtained by slowly evaporating the sample on one side of the ATR plate under a stream of nitrogen (36). The ATR plate was then sealed in a universal sample holder.

**Secondary Structure Analysis**—The sample on the ATR plate was rehydrated by flushing D₂O-saturated N₂ for 2 h at room temperature. 512 scans were averaged for each measurement. The determination of the secondary structure was based on the shape of the amide I band (1600–1700 cm⁻¹), which is sensitive to the secondary structure (37). The analysis was performed on the amide I region of deuterated samples because the H/D exchange allows differentiation of the α-helical secondary structure from the random secondary structure whose absorption band shifts from about 1655 cm⁻¹ to about 1642 cm⁻¹ (38). A combination of Fourier self-deconvolution and a least squares iterative curve fitting was applied to narrow and to quantify the area of the different components of amide I band between 1700 and 1600 cm⁻¹ (39).

To avoid the introduction of artifacts because of the self-deconvolution procedure, the fitting was performed on the non-deconvoluted spectrum. The proportion of a particular structure was computed as the sum of the area of all of the fitted Lorentzian bands having their maximum in the frequency region where that structure occurs divided by the total area of the amide I. The frequency limits for each structure were assigned according to theoretical (40) or experimental (41) data: 1662 to 1645 cm⁻¹, α-helix; 1689 to 1682 and 1637 to 1613 cm⁻¹, β-sheet; 1644 to 1622 cm⁻¹, 310-helical; 1682 to 1682.5 cm⁻¹, β-turns.

**Orientation of the Secondary Structures**—The orientation of different secondary structural states was determined as described previously (42, 43). Spectra were recorded with the incident light polarized parallel and perpendicular with respect to the incidence plane. Dichroism spectra were computed by subtracting the perpendicular polarized spectrum from the parallel polarized spectrum. The subtraction coefficient was chosen so that the area of the lipid ester band at 1740 cm⁻¹ equals zero on the dichroism spectrum, to take into account the difference in the relative power of the evanescent field for each polarization and also the differences in film thickness as described previously (44).

An upward deviation on the dichroism spectrum indicates a dipole oriented preferentially near a plane to the ATR plate. Conversely, a downward deviation on the dichroism spectrum indicates a dipole oriented closer to the plane of the ATR plate (36). The dichroic ratio R_{ATR} is defined as the ratio of the amide I area recorded for the parallel polarization (A₁) and perpendicular polarization (A₂) as shown in Equation 1.

\[ R_{ATR} = A_1 / A_2 \]  

(Eq. 1)

In ATR, the dichroic ratio for an isotropic sample R_{iso} is different from unipolarity and is computed on the basis of the area of the lipid ester band (1762–1700 cm⁻¹). As described previously, the presence in the sample of a fraction x of oriented helices and a fraction 1 – x of randomly oriented dipoles results in an experimental dichroic ratio R_{ATR} whose value is between the value of oriented helices dichroic ratio Ra and of randomly oriented dipoles R_{iso}.

\[ R = R_{iso} x + R_a (1-x) \]  

(Eq. 2)

In an α-helical structure, the amide I dipole is oriented 27° (44) with respect to the helix axis. The mean orientation of the helix axis with respect to the membrane was estimated as described previously (for review, see Ref. 45).

**HID Exchange Kinetics**—20 μg of reconstituted MRP1 was deposited on a germanium plate as described above. Nitrogen was saturated with D₂O by bubbling in a series of three vials containing D₂O. Before starting the deuteration, 10 spectra of the sample were recorded to test the stability of the measurement. When the D₂O-saturated N₂ flux, at a flow rate of 100 ml/min, was connected to the sample. For each kinetic point, 24 scans were recorded and averaged at a resolution of 2 cm⁻¹. The signal from the atmospheric water was subtracted as described by Goormaghtigh and Russchaert (46). As described previously (40), deuteration of protein side chains induces modifications in the amide I (1700 to 1680 cm⁻¹) and amide II (1600 to 1500 cm⁻¹) regions. Several parameters modulate their contribution including the ionization state of the carboxylic amino acids and the fraction of deuterated and undeuterated amino acid side chains for every spectrum of the kinetics. We used homemade software, which can compute the contribution of the amino acid side chains as a function of the extent of deuteration (47). The area of the amide II, characteristic of the 8(N–H) vibration, was obtained by integration between 1596 and 1502 cm⁻¹. For each spectrum, the area of the amide II was divided by the corresponding lipid ν(C=O) area to take into account small but significant variations in the total intensity due to the presence of D₂O which induced swelling of the sample layer at the beginning of the kinetics (39). This ratio expressed in percentage was plotted versus deuteration time. The value corresponding to 0% of deuteration is defined by the amide II/lipid ratio obtained before deuteration. The 100% value corresponds to a zero absorption in the amide II region, observed for full deuteration of the protein.

**Measurements of ATPase Activity**—ATPase activity of reconstituted MRP1 was measured by following the release of the inorganic phosphate using a colorimetric method (48). Proteoliposomes (4 μg of MRP1) were incubated in 10 mM Hepes, pH 7.4, 6 mM MgCl₂, 3 mM ATP or AMP-PNP for 10 min at 37 °C before trypsin digestion was performed. Trypsin was added to the mixture (trypsin/MRP1 ratio is 1:400, w/v) and incubation carried out at 25 °C. After the desired times of incubation, aliquots were removed from the mixture, and the reaction was stopped by adding a trypsin inhibitor solution (TLCK) to 50 μg/ml. Zero time controls were obtained by adding trypsin inhibitor before adding trypsin. The peptide fragments were separated through a Tri-Tricine polyacrylamide gel (16.5% T, 3% C) and visualized by silver staining.
Effects of different ligands on trypsin activity were assayed using BAPNA as substrate. The tryptic activity was tested at room temperature in 200 μl of medium containing 10 mM Hepes, pH 7.4, 10 μg of trypsin, 0.5 mM BAPNA with or without ligands. The enzymatic activity of trypsin was determined by measuring the absorbance of p-nitroanilide at 405 nm. This absorbance was recorded immediately after mixing and continued every minute for 10 min and every 2 min until 30 min, on a Labsystems iEMS Reader MF spectrophotometer.

RESULTS

The aim of the present study was to investigate how GSH affects the structure of MRP1 at different steps of the transport cycle, i.e. the drug-binding step and the ATP-binding and hydrolysis step. Experiments are conducted in the presence of two anthracycline derivatives that either do accumulate or do not accumulate in resistant cells. Doxorubicin, a widely clinically used anticancer agent, is actively extruded from cells overexpressing MRP1 (2–4). In contrast, a doxorubicin analogue, 3′-(3′-methoxymorpholino-doxorubicin (FCE), accumulates identically in drug-sensitive and -resistant cells overexpressing MRP1, demonstrating that this compound is not transported by MRP1 (49). Conformational changes induced by binding and hydrolysis of ATP are analyzed in the presence of MgAMP-PNP and MgATP. The use of MgAMP-PNP, a nonhydrolyzable analog of MgATP, allows discrimination between the influence of nucleotide binding and nucleotide hydrolysis on MRP1 structure.

The study is performed on purified MRP1 reconstituted into liposomes of asolectin as described previously (34). The reconstituted MRP1 retains its ATPase and transport activity. The orientation of MRP1 into liposomes was investigated by comparison of its ATPase activity before and after permeabilization of liposomes in the presence of 2 mM Chaps. This Chaps concentration has been shown previously to permeabilize liposomes (34, 50). We observed that the ATPase activity was not enhanced by addition of 2 mM Chaps indicating that MRP1 is inserted into liposomes in an inside-out configuration (34).

Secondary Structure Analysis—The infrared spectrum of MRP1 alone, in the 1800 to 1400 cm⁻¹ region, is shown in Fig. 1. The lipid ester (C=O) band is located in the 1800 to 1700 cm⁻¹ region. The amide I band due to the (C=O) vibration of the peptide bonds is located between 1700 and 1600 cm⁻¹ and is highly sensitive to the secondary structure. The 1600 to 1500 and 1500 to 1400 cm⁻¹ bands represent unexchanged and exchanged amide II, respectively, corresponding to the (N–H) and (N–D) amide bonds.

The secondary structure of MRP1 in the absence or in the presence of ligands was determined by Fourier deconvolution and curve-fitting analysis of the amide I band of a deuterated sample as described previously (39). The proportions of different secondary structures are shown in Table I and indicate that drug and GSH binding in the absence or in the presence of ATP or AMP-PNP do not modify MRP1 secondary structure.

Orientation in MRP1—Spectra of MRP1 are recorded with parallel and perpendicular polarized light (Fig. 2, A and B). Dichroic spectra are obtained by subtracting the perpendicular
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TABLE I
MRP1 secondary structure composition determined in the absence and in the presence of substrates

| Substrate       | α-Helix | β-Sheet | β-Turn | Random |
|-----------------|---------|---------|--------|--------|
| None            | 42 ± 3  | 29 ± 3  | 12 ± 4 | 17 ± 6 |
| GSH             | 42 ± 5  | 29 ± 3  | 14 ± 7 | 19 ± 7 |
| DOX             | 43 ± 4  | 31 ± 2  | 12 ± 1 | 15 ± 1 |
| FCE             | 39 ± 6  | 29 ± 2  | 14 ± 5 | 19 ± 4 |
| DOX + GSH       | 41 ± 5  | 30 ± 6  | 12 ± 6 | 17 ± 1 |
| DOX + GSH + AMP-PPN | 41 ± 2 | 29 ± 2 | 14 ± 7 | 15 ± 4 |
| FCE + GSH + AMP-PPN | 39 ± 3 | 31 ± 5 | 10 ± 3 | 19 ± 1 |
| DOX + GSH + AMP-PPN | 40 ± 3 | 32 ± 5 | 11 ± 5 | 17 ± 3 |
| FCE + GSH + ATP  | 39 ± 5  | 29 ± 3  | 11 ± 2 | 19 ± 6 |
| FCE + GSH + MgATP| 40 ± 5  | 32 ± 4  | 10 ± 1 | 17 ± 7 |

* MRPI/GSH molar ratio = 1.3.
* MRPI/drug molar ratio = 1:14.
* MRPI/nucleotide molar ratio = 1:6.

**Experimental Procedures.**

The mean orientation of the transmembrane helices obtained from the dichroic ratios is calculated around 42° with respect to the normal to the germanium plate. Considering an orientation of the amide I dipole of 27° as described under "Experimental Procedures." The mean orientation of the helices is not significantly modified by the presence of different ligands (Table II). Simultaneous binding of FCE, GSH, and MgATP modifies the mean orientation of the helices.

**Kinetics of Deuteration—** At constant experimental conditions (pH and temperature), the rate of hydrogen/deuterium exchange is related to the solvent accessibility to the NH amide groups of the protein, which in turn is related to the tertiary structure of the protein and to the stability of a specific conformational state as well as the secondary structure of the protein. The IR analysis of the secondary structure of MRP1 in the presence of different ligands shows that its secondary structure is not modified, differences in H/D exchange rate will reflect changes in the tertiary structure of the protein and/or in the stability of a specific conformational state. Amide hydrogen exchange was followed by monitoring the amide II absorption peak (maximum at 1544 cm⁻¹) as a function of time of exposure to D₂O-saturated N₂ flow (Fig. 1). The decreasing area of amide II computed between 100 and 0% (see "Experimental Procedures") is reported in Figs. 3 and 4. In the absence of ligand, and after 2 h of deuteration, 39% of the amide hydrogens remain unexchanged. These amide hydrogens are poorly accessible to the aqueous medium or involved in stable secondary structures. GSH binding strongly affects the protein accessibility and/or the stability of the conformational state of the protein because the percentage of unexchanged amide hydrogens increased from 39 to 60% (Fig. 3). This means that GSH binding mediates a conformational change that affects 21% (321 amino acid residues) of the total number of MRP1 amino acid residues. Binding of FCE or DOX decreases the number of unexchanged hydrogens from 39 to ~28%, indicating that a conformational change has occurred that increases the accessibility of the protein toward the aqueous phase and/or decreases the stability of the conformational state of the protein (Fig. 3). 168 amino acids residues are implicated in this conformational change. In the presence of GSH and drug, no significant change of the exchange rate is observed compared with the protein alone, no matter what kind of drug was utilized (Fig. 3). Around 40% of the amino acid residues remain unexchanged after 2 h of deuteration. This suggests that after simultaneous binding of GSH and drugs, MRP1 behaves as in (36, 43), are calculated as described under "Experimental Procedures."
the means of three experiments.

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\[ H(t) = \sum a_i \exp(-t/T_i) \]  
(Eq. 3)

where \( a_i \) is the proportion of each amide group with identical \( T_i \) values.

The large number of protons makes it impossible to obtain the individual rate constants. One approach to this problem is to choose arbitrarily a number of exponentials to fit the exchange curves. We have chosen three exponentials of amide groups, characterized by their period \( T_i \) (\( i = 1-3 \)) and their proportion \( a_i \). In order to compare the proportion \( a_i \) of each amide group for all the experimental curves, the same \( T_i \) values were used for all the fittings. These periods were chosen as follows: \( T_1 = 1 \) min for the very fast exchanging protons \( (a_1) \), \( T_2 = 9 \) min for the intermediate \( (a_2) \), and \( T_3 = 666 \) min for the very slow exchanging protons \( (a_3) \) (Table III). In the absence of any ligand, \( -33\% \) of the amide hydrogens was exchanged for deuterium within 1 min, and an additional 28\% after 9 min. The remaining 39\% hydrogens exchanged much slower and represent the very inaccessible regions of MRP1. In the presence of GSH, a large fraction of the very fast exchanging protons became inaccessible to the aqueous medium (about 21\% or 321 amino acids). Drug binding has the opposite effect. Very slow exchanging protons are now very quickly exchanged with deuterium (about 11\% or 168 amino acids). The simultaneous presence of GSH and drugs results in an MRP1 conformation for which exchange kinetics is similar to that of the protein alone. In the presence of ATP or nonhydrolyzable analog AMP-PNP, the kinetics are quite dependent on the nature of the drug. When doxorubicin is bound, subsequent ATP binding exposes the protein to its aqueous environment, and the slow exchanging population of protons decreases with a concomitant increase of the fraction of protons exchanging at a fast rate (Table III). When ATP is hydrolyzed, the slow exchanging population of protons decreases with a concomitant increase of the fraction of protons exchanging at intermediate rate (Table III). The situation is quite different with FCE, a non-trans-

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In contrast, when FCE and GSH were simultaneously bound, addition of MgATP led to an increase of the percentage of unexchanged hydrogens from 40 to 53\% (Fig. 4). As observed for DOX, this decrease of the accessibility of the protein to the aqueous phase and/or increase of the stability of the conformational state is due to the ATP binding, rather than ATP hydroly-

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Limited Proteolysis of MRP1 with or without Ligands—Tryp-

sin cleavage site accessibility has been successfully used to investigate ligand-induced conformational changes of various proteins, including ABC transporters (P-glycoprotein, MPM70).
(53–55) and ATPases (K⁺, H⁺-ATPase) (56). In our experiments, reconstituted MRP1 was incubated (10 min) with different substrates before being subjected to trypsin digestion. At various times, aliquots were removed from the mixture and added to the trypsin inhibitor solution. The trypsin digests were analyzed on Tris-Tricine gels.

The effect of ligands on trypsin activity is assayed using BAPNA as substrate (Table IV). The trypsin to protein ratio is adjusted in order to maintain the same trypsin activity under various circumstances. Therefore, changes in the trypsin digestion profile cannot be attributed to differences in trypsin activity but reflect a different susceptibility of MRP1 to tryptic cleavage upon ligand binding.

Fig. 5 (lanes 2–6) shows that incubation of MRP1 with trypsin resulted in a gradual disappearance of the full-length ~190-kDa MRP1 and concomitant appearance of peptide fragments with approximate molecular masses of 130, 115, 75, and 60 kDa. These fragments have been identified previously using antibodies raised against defined epitopes corresponding to different regions of MRP1 (21, 24, 28, 29, 57). The 115 and 75-kDa fragments correspond to the cleavage at the trypsin-sensitive site in the cytoplasmic region linking NBD1 to MSD2 (L1) (Fig. 6). The 115-kDa fragment encodes sequences that include MSD0, L0, MSD1, and NBD1; the 75-kDa fragment corresponds to the COOH-terminal fragment containing MSD2 and NBD2. The exact location of the second trypsin cleavage site generating the 130- and 60-kDa fragments remains contradictory and is assigned either in the cytoplasmic loop (L0) linking MSD0 to MSD1 or in the cytoplasmic loop CL4 (Fig. 6). Although identification of the exact cleavage site responsible for the generation of the 130 and 60 kDa is complicated by the attachment of N-linked oligosaccharide chains, the 130-kDa band must contain NBD1 and NBD2 (24, 57). Therefore, the 60-kDa fragment represents either MSD0 and part of L0 cytoplasmic loop or MSD0, L0 cytoplasmic loop, and the two first transmembrane helices of MSD2. These fragments are subsequently digested by prolonged trypsin incubation.

GSH has a marked protective effect against digestion of MRP1 by trypsin (Fig. 5, lanes 7–11). This is consistent with a conformational change affecting the two sensitive trypsin sites that are less exposed to the solvent. GSH binding increases also the stability of fragments at 130, 115, 75, and 60 kDa suggesting that further degradation of these fragments in the connecting loops is also prevented. In contrast, the presence of drugs (DOX or FCE) (Fig. 7, lanes 7–16) does not modify the pattern of digestion obtained for the protein alone (Fig. 7, lanes 2–6) indicating that the protein is just as sensitive to trypsin in the drug-bound state as the protein alone. In the presence of both GSH and DOX (Fig. 8, lanes 7–11) or GSH and FCE (Fig. 8, lanes 12–16), the whole protein is less efficiently digested compared with the protein alone (Fig. 8, lanes 2–6). The digestion profile is very similar to that obtained with GSH alone (Fig. 5, lanes 7–11) confirming that only GSH affects the structure of the cytosolic domains.

In the presence of MgAMP-PNP, a nonhydrolyzable analog of MgATP, DOX, and GSH (Fig. 9, lanes 12–16), the pattern of digestion is drastically modified compared with the one of protein alone (Fig. 9, lanes 2–6). The sensitivity to trypsinolysis for the full-length protein and the 130-, 115-, 75-, and 60-kDa fragments is increased, as judged by their rapid and total disappearance on the gel. Increased exposure facilitates the attack of trypsin on the proteolytic site(s) located in these regions. The same digestion profile is observed when DOX (Fig. 9, lanes 12–16) is replaced with FCE (Fig. 9, lanes 7–11). When MgAMP-PNP is replaced with MgATP, a similar enhanced sensitivity to the trypsin is observed (Fig. 10), demonstrating that MgATP binding, rather than MgATP hydrolysis, induces a conformational change that makes the cytoplasmic loops more accessible to the solvent.

**ATPase Activity Measurements**—The ATPase activity of reconstituted MRP1 is about 8 nmol/mg/min. FCE or DOX binding does not modify significantly the rate of ATP hydrolysis (Fig. 11), whereas 21% stimulation is observed in the presence of 3 mM GSH. No additional stimulatory effect is detected when drugs and GSH are bound simultaneously to MRP1. Hooijberg et al. (58) have reported a similar stimulation of the ATPase activity.

### Table III

| Ligand      | α₁ | α₂ | α₃ |
|-------------|----|----|----|
| None        | 33 | 28 | 39 |
| GSH         | 138| 41 | 60 |
| DOX         | 720| 113| 27 |
| FCE         | 42 | 44 | 42 |
| DOX + GSH   | 505| 42 | 62 |
| FCE + GSH   | 366| 38 | 39 |
| DOX + GSH + AMP-PNP | 566| 50 | 50 |
| DOX + GSH + ATP | 459| 52 | 32 |
| FCE + GSH + AMP-PNP | 360| 50 | 51 |
| FCE + GSH + ATP | 306| 42 | 53 |

### Table IV

| Ligand                                      | Remaining trypsin activity % |
|---------------------------------------------|------------------------------|
| Control                                     | 100                          |
| 20 µM anthracycline                        | 100                          |
| 3 mM GSH                                    | 60                           |
| 3 mM GSH + 20 µM anthracycline              | 80                           |
| 3 mM GSH + 20 µM anthracycline + 3 mM MgAMP-PNP | 80                           |
| 3 mM GSH + 20 µM anthracycline + 3 mM MgATP  | 51                           |

**Fig. 5. Electrophoretic pattern of MRP1 peptides after trypsin digestion in the absence or presence of GSH.** MRP1 was treated without (lanes 2–6) or with 3 mM GSH (lanes 7–11) for 10 min at 37°C, followed by trypsin digestion at a 1:400 (w/w) trypsin/protein ratio at room temperature for the times indicated. The reaction was stopped by addition of trypsin inhibitor (TLCK), and the trypsin fragments were separated on a Tris-Tricine gel and visualized by silver staining. Each lane contains 1.5 µg of protein. Lane 1 shows the protein in the absence of trypsin.
activity by GSH in MRP1-containing membrane vesicles; transported drugs vincristine and daunorubicin do not activate the enzyme.

DISCUSSION

GSH is required for MRP1 to confer cell resistance to chemotherapeutic drugs including anthracyclines, vinca alkaloids, and etoposide (7–14). How GSH facilitates the transport of these drugs is not yet known, but it is clearly not related to the sulfhydryl reducing capacity of this tripeptide because S-methyl GSH and other short chain alkyl derivatives of GSH also facilitate transport (9, 18). The proposal that these anticancer drugs are pumped out of cells by MRP1 via a co-transport mechanism with free GSH is still a matter of debate (6, 9, 14). Structural information about the effect of GSH binding to MRP1 at different stages of the transport cycle is certainly required for understanding this complex mechanism. As this information is not available, we decided to investigate the conformational changes induced upon GSH interaction with the protein in the absence or in the presence of amphiphilic drugs (i.e. anthracyclines) and ATP, in order to characterize structural intermediate states involved in the MRP1-mediated transport process.

We combined different experimental approaches to gain structural information about the cytosolic and membrane-embedded domains of MRP1. First, quantitative global information about the conformational changes occurring in the protein was obtained by monitoring the amide hydrogen exchange. Second, IR-polarized attenuated total reflection was used to gain information about the orientation of MRP1 transmembrane domains. Third, trypsin digestion and ATPase activity assays allowed us to investigate structural changes of the cytosolic domains.

Several conclusions can be drawn from this study. First, MRP1 does not undergo detectable secondary structure changes upon interaction with its substrates (Table I).

Does ligand binding affect MRP1 tertiary structure? A change in the tertiary structure is supported by the H/D exchange rate measurements demonstrating that after drug (DOX and FCE) binding, the accessibility toward the solvent is increased and/or the stability of the conformational state is decreased (Fig. 3 and Table III). 168 amino acid residues are involved in this conformational change. This tertiary structure...
ATPase activity. ATP hydrolysis is presented as % of the control

MRP1 was treated without (lanes 2–6) or with 20 μM FCE + 3 mM GSH + 3 mM MgAMP-PNP (lanes 7–11) or 20 μM DOX + 3 mM GSH + 3 mM MgAMP-PNP (lanes 12–16) for 10 min at 37 °C, followed by trypsin digestion at a 1:400 (w/w) trypsin/protein ratio at room temperature for the times indicated. The reaction was stopped by addition of trypsin inhibitor (TLCK), and the tryptic fragments were separated on a Tris-Tricine gel and visualized by silver staining. Each lane contains 1.5 μg of protein. Lane 1 shows the protein in the absence of trypsin.

**Fig. 10.** Electrophoretic pattern of MRP1 peptides after trypsin digestion in the absence or presence of anthracyclines, GSH, and MgAMP-PNP. MRP1 was treated without (lanes 2–6) or with 20 μM FCE + 3 mM GSH + 3 mM MgAMP-PNP (lanes 7–11) or 20 μM DOX + 3 mM GSH + 3 mM MgAMP-PNP (lanes 12–16) for 10 min at 37 °C, followed by trypsin digestion at a 1:400 (w/w) trypsin/protein ratio at room temperature for the times indicated. The reaction was stopped by addition of trypsin inhibitor (TLCK), and the tryptic fragments were separated on a Tris-Tricine gel and visualized by silver staining. Each lane contains 1.5 μg of protein. Lane 1 shows the protein in the absence of trypsin.

**Fig. 11.** Effect of anthracyclines and GSH on the MRP1 ATPase activity. ATP hydrolysis is presented as % of the control ATPase activity assayed without ligand (8 nmol min⁻¹ mg⁻¹ of protein). A concentration of 20 μM of each anthracycline and 3 mM GSH was used. The values represented are the means of three experiments ± S.E.

change does not affect the conformation of the cytosolic domains as illustrated by limited trypsin digestion experiments (Fig. 7). This idea is also supported by the fact that ATP hydrolysis is not significantly modified in the presence of drugs (Fig. 11). Consequently, the conformational change detected using H/D exchange may reflect a restructuring of the protein in its membrane-embedded domain where the drug(s)-binding site(s) are located. The fact that drug binding does not affect the orientation of the transmembrane helices may suggest a rotation or translation movement of the transmembrane helices that would explain H/D exchange modifications. Interestingly, cysteine cross-linking experiments on P-glycoprotein, another related ABC transporter, have shown a reorganization of the membrane-embedded helices after drug binding (59). However, in the case of Pgp, the conformational change mediated by DOX and FCE binding to the membrane helices is transmitted to the cytosolic domain and stimulates the ATPase activity (60). These data suggest that, in the case of MRP1, drug binding is not sufficient to allow the cross-talk between the membrane and the cytosolic domains.

A drastic modification of the accessibility and/or the stability of the conformational state of MRP1 is observed in the presence of GSH; the proportion of unexchanged hydrogens increased from 39 to 60% (Fig. 3 and Table III). This conformational change affects, at least partly, the conformation of the cytosolic domain as indicated by the protection of the trypsin cleavage sites (Fig. 5) and could be required to stimulate the ATPase activity (Fig. 11). These data strongly suggest that GSH allows the coupling between drug transport and ATP hydrolysis. This coupling cannot be achieved in the drug-bound state. It has been reported previously that GSH binding greatly reduces $K_m$ and increases $V_{max}$ for drugs, suggesting a restructurin of the transmembrane helices that might expose residues to the drug-binding site and increase the affinity of the protein for the drug (9, 14). Our data suggest that this change in affinity is related to rotation movements of transmembrane helices along their own axis because the global tilt of the transmembrane helices is not modified (Fig. 12, State III). These rotation movements would reorient the amino acids involved in drug binding in such a way that drug binding is enhanced.

Incubation of MRP1 with both GSH and drugs generates a conformation that is distinct from the conformation stabilized by each ligand. The level of H/D exchange is equivalent to the one obtained for the protein in the absence of ligand (Fig. 3 and Table III). Does this mean that GSH binding somehow compensates the effect of drug binding and brings the protein back into its initial conformation? This seems unlikely because limited proteolysis of MRP1 shows that simultaneous drug and
Conformational changes induced by binding and hydrolysis of ATP are analyzed in the presence of MgAMP-PNP and MgATP. The use of MgAMP-PNP, a non-hydrolyzable analog of MgATP, allows discrimination between the influence of nucleotide binding and nucleotide hydrolysis on MRP1 structure. The major structural reorganization of MRP1, during drug translocation, occurs after ATP binding, rather than ATP hydrolysis. ATP binding reorganizes MRP1 structure in a conformation that has a decreased stability and/or increased accessibility to the aqueous phase (Fig. 4 and Table III). This conformational change affects at least partly the cytosolic domain as demonstrated by increased sensitivity to trypsinolysis (Fig. 9). No significant change in the stability of this conformational state and/or the global accessibility of the protein (Fig. 4 and Table III) nor of the accessibility of the trypsin cleavage site (Fig. 10) is observed after ATP hydrolysis. It is possible that hydrolysis of ATP induces the rotation of transmembrane helices in the plane of the membrane. This movement does not require a change in the mean tilt of the transmembrane domains. The rotation of the transmembrane helices upon ATP hydrolysis has been demonstrated in the case of Pgp, by cysteine cross-linking studies (61). This rotation exposes different residues to the drug binding pocket, reducing the drug binding affinity. Furthermore, it has been shown previously that ATP hydrolysis, rather than ATP binding, reduces the affinity of MRP1 for its substrate (62).

It is also interesting to note that two-dimensional crystals of Pgp have revealed that ATP binding, not hydrolysis, drives the major conformational change associated with substrate translocation (63).

Binding of FCE, a non-transported drug, and GSH does not impair ATP binding and hydrolysis but affects the nature of the conformational change occurring during these steps of the transport process. ATP binding mediates a conformational change that decreases the accessibility of the protein to the aqueous phase and/or increases the stability of the conformational state (Fig. 4 and Table III). Subsequent hydrolysis of ATP does not modify the stability of this conformational state and/or the global accessibility of the protein. Despite this global decrease of accessibility, trypsin digestion reveals an increase of the accessibility of the cytosolic domain to the aqueous medium (Figs. 9 and 10). Consequently, the decrease in the global accessibility observed in H/D exchange upon binding and hydrolysis of ATP must affect the membrane domains. Interestingly, analysis of the dichroic spectra has revealed that a significant change in the mean orientation of the transmembrane helices occurs only upon ATP hydrolysis. This supports the idea that after ATP binding, the reorganization of the transmembrane helices involves translational movements, whereas ATP hydrolysis modifies the tilt of the transmembrane helices. This reorganization inhibits the passage of the drug from its high affinity binding site to its low affinity binding site, and the drug remains bound to the protein.

**Translocation Model Proposed for MRP1**—A model describing the MRP1-mediated mechanism of drug transport is proposed in Fig. 12. This model is based on the biochemical evidence that drugs binding to the protein occur from the inner leaflet of the bilayer (26–32). In contrast, GSH-binding site is accessible from the cytosol (64). ATP hydrolysis reduces the affinity of MRP1 for its substrate (61), which allows its release into the extracellular medium (13, 18). In its initial state (Fig. 12, State I), the high affinity drug-binding site is exposed to the membrane, and the high affinity GSH-binding site is oriented toward the cytosolic domain. The protein-drug interaction causes a structural modification in the membrane-spanning domains that may involve rotation or translation movements.

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**Fig. 12. Schematic representation of different structural states involved in GSH- and ATP-dependent drug transport mediated by MRP1.** The transmembrane domains are represented by cylinders. The ellipse, hexagon, and square represent the different conformations of the cytosolic domain (NBDs are not represented). MRP1 has binding sites for drugs (circle with hatched lines) and for GSH (opened circle). These two sites may exist in a high (circle with hatched lines and opened circle) or low (square with hatched lines and opened square) affinity state for their respective ligands (shaded for drug and open for GSH). Drug and GSH are represented as closed diamond and closed circle, respectively. In and out refer to the inside and outside of the cell. Arrows are representation of putative translation and rotation movements of transmembrane helices. State I, the high affinity binding sites of drug and GSH are exposed to the inner leaflet of the bilayer for the drug and toward the cytosol for GSH. State II, drug binding, in the absence of GSH, induces a conformational change in the transmembrane domains which involves rotational and/or translational movements. This conformational change is not transmitted to the cytosolic domains. State III, subsequent binding of GSH induces a structural reorganization affecting both the cytosolic domains and the transmembrane domains. The conformational change occurring in the cytosolic domain stimulates ATP hydrolysis. The reorganization of the membrane-embedded domains of MRP1 involves rotational movements that reorient residues located in the transmembrane helices and enhances drug binding. State IV, ATP binding increases the accessibility of the cytosolic domain to the aqueous phase. State V, ATP hydrolysis induces the rotation of the transmembrane helices that decreases the affinity for the ligands and allows their release into the external medium. State IV′, when a non-transported drug is bound to the protein, ATP binding reorganizes the transmembrane domains through translational movements. ATP hydrolysis (State V′) modifies the tilt of the transmembrane helices. Such a conformation limits the accessibility toward the aqueous phase and restricts the translocation of the drug. Our structural data provide no information about the mechanism by which the protein comes back to its initial state.

GSH binding leads to a proteolytic pattern different from the one obtained with the protein alone (Fig. 8). In conclusion, in the presence of GSH and drug the protein adopts a conformation that does not correspond to that of the protein alone but both conformations are characterized by the same stability and/or global accessibility toward the solvent.
(Fig. 12, **State II**). Subsequent binding of GSH (Fig. 12, **State III**) modifies the structure of the membrane-embedded domain and the ATP-binding sites located in the cytosolic domain. The restructuring of the transmembrane helices may be due to rotation movement in the membrane but not to a reorientation of these domains. The cytosolic domain adopts a conformational change which opens a pathway through which translocation occurs, a prerequisite for the release of the substrates into the extracellular medium. ATP hydrolysis (Fig. 12, **State V**) would change the affinity of MRP1 for substrate through helix rotation in the drug-binding domain. Consequently, they are released into the external medium. Occupation of the binding site by a non-transported drug (FCE) (Fig. 12, **State IV** and **State V**) may prevent it from gaining access to the opposite side of the membrane and thereby prevent transport.

In conclusion, we have characterized several structural intermediates involved in MRP1-mediated drug transport. It is worth mentioning that structural changes, first identified by using the IR approach described here, have been shown to be consistent, respectively, with a three-dimensional map and two-dimensional crystals for H^+ATPase (65, 66) and Pgp (63, 67).

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