Structure, Orientation, and Conformational Changes in Transmembrane Domains of Multidrug Transporters

CATHERINE VIGANO,† LILIANA MANCIU, AND JEAN-MARIE RUYSSCHAERT*

Laboratory for Structure and Function of Biological Membranes, Structural Biology and Bioinformatics Center, Free University of Brussels, Brussels, Belgium

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ABSTRACT

Multidrug transporter proteins promote the active transmembrane efflux of noxious drugs, thereby decreasing their accumulation in the intracellular medium and reducing their therapeutic efficiency. Expression of such proteins drastically reduces the efficiency of chemotherapeutic treatments against cancer and various infectious diseases. To overcome major difficulties related to the crystallization of membrane proteins, other experimental approaches have been developed to gain information on the structural changes involved in drug transport. We examine here and illustrate with a few examples how infrared and fluorescence spectroscopy can provide new insights into the structure of the membrane domains of multidrug transporters in particular. Such domains contain the drug-binding site(s) and mediate the passage of substrates across the cell membrane.

Introduction

Many, if not all, pathogenic microorganisms as well as human cancer cells have developed several resistance mechanisms in response to the exposure to chemotherapeutic agents. This phenomenon is the major reason that treatment of infectious diseases or malignant tumors may fail. Ordinarily, resistance is specific to a single class of cytotoxic agents and is referred to as "specific drug resistance" or SDR. Perhaps of greater concern than SDR is the development of cells that are cross-resistant to several structurally and functionally unrelated compounds. This phenomenon has been termed "multidrug resistance" or MDR. It was initially discovered in the treatment of mammalian tumor cells,[1] but nowadays, it is known to play an important role in drug resistance of a broad range of pathogenic bacteria[2] and parasitic protozoa.[3] MDR phenotype may be due to the expression of different MDR transporter proteins. These transporters promote the active pumping out or transmembrane efflux of noxious drugs thereby decreasing their accumulation in the intracellular medium and reducing their therapeutic efficiency. MDR transporters are members of a limited number of families. On the basis of the energy source, these transporters can be divided into two major classes (Figure 1). The first class is composed of primary active transporters that are energized by ATP hydrolysis.[4] These primary transporters are represented by the superfamily of ATP-binding cassette (ABC) transporters. These proteins are characterized by the presence of two extramembrane nucleotide-binding domains or NBDs, whose catalytic activity drive the transport process. The second class consists of secondary active transporters that mediate drug efflux in a symport or antiport exchange reaction with protons or sodium ions.[5] ABC transporters play an important role in drug efflux in eukaryotic cells, including human cancer cells. In particular, the expression of P-glycoprotein (Pgp)[6] and the MDR-associated protein, MRP1,[7] is of great medical importance. In prokaryotic organisms, the secondary transporters are predominantly active in drug extrusion. The lactococcal ABC multidrug transporter LmrA however focuses interest because it is the bacterial homologue of Pgp. When expressed in human cells, this protein is indeed able to complement Pgp because it confers the same MDR phenotype as the human transporter.[8]

Several questions have dominated the MDR field of research. For example, how do MDR transporters bind and transport multiple structurally unrelated substrates? How do they coupled energetic release with drug efflux? How can we rationally design efflux inhibitors? In fact, all of these questions require the knowledge of the structures of representative pumps and the elucidation of the conformational changes that accompany drug translocation. In particular, the structure of the transmembrane domain of these transporters merits attention because it contains the drug-binding site[9−11] and mediates the passage of substrates across the cell membrane. To mediate drug binding in the membrane and subsequent release in the external medium, this membrane-embedded domain must pass through at least two configurations: a high-affinity membrane-facing drug-binding site and a low affinity for the substrate, outside-facing site, for drug release. Consequently, conformational changes must oc-

Catherine Vigano was born in 1975 in Belgium. She received her master degree (1998) and Ph.D. degree (2002) in bioengineering from the Free University of Brussels. She is a postdoctoral researcher of the National Fund for Scientific Research (FNRS, Belgium) since 2002. Her research has centered on multidrug resistance proteins such as LmrA, P-glycoprotein, and LmrP.

Liliana Manciu was born in 1973 in Romania. She received her master degree (1997) and Ph.D. degree (2003) in chemistry from the Free University of Brussels, Belgium. Her research was mainly focused on the structural characterization of MRP1, a protein involved in cancer resistance to chemotherapy. Presently, she is employed as a Scientific Affairs Manager at the Amsterdam group (Brussels), an alliance of Europe's leading producers of beers, wines, and spirits.

Jean-Marie Ruysschaert was born in 1939 in Belgium. He received his master degree (1961) and Ph.D. degree in chemistry (1965) from the Free University of Brussels. He worked as a postdoctoral researcher at the Cambridge University (U.K.), Weizmann Institute of Sciences (Israël), and Carnegie Institute (Baltimore, MD). He then joined the Department of Chemistry at the Free University of Brussels where he created his laboratory for Chemistry and Physics of Macromolecules at the Interfaces. He obtained a position as associate professor in 1986 and became professor in 1991. Since 1999, he is the Director of the Center for Structural Biology and Bioinformatics (Brussels, Belgium).

* To whom correspondence should be addressed: Structure et Fonction des Membranes Biologiques, Université Libre de Bruxelles, CP206/2, Bd du Triomphe, 1050 Brussels, Belgium. Fax: 003226505382. E-mail: jmruyss@ulb.ac.be.

† Postdoctoral researcher for the National Fund for Scientific Research (FNRS), Belgium.

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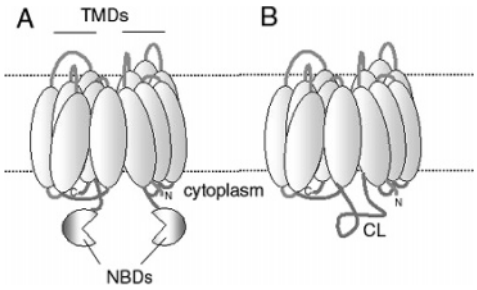

FIGURE 1. Structural organization of ABC transporters (A) and secondary drug transporters (B) (adapted from Bolhuis, H.; van Veen, H. W.; Poolman, B.; Driessen, A. J.; Konings, W. N. Mechanisms of multidrug transporters. *FEMS Microbiol. Rev.* **1997**, *21*, 55−84). (A) Two transmembrane domains (TMD), each consisting of six putative α-helical segments (depicted as ellipses) are present in the membrane. The NBDs are located at the cytoplasmic surface of the membrane. (B) Transmembrane α-helical segments are indicated as ellipses. The carboxy (C) and amino (N) termini and the large central loop (CL) are located in the cytoplasm.

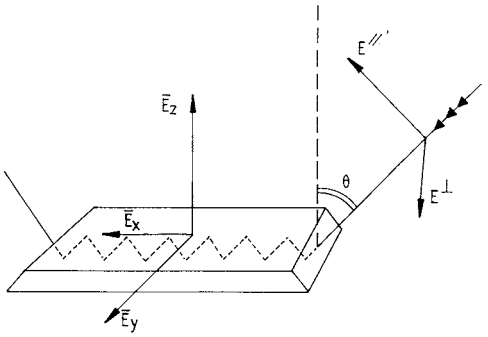

FIGURE 2. Schematic representation of the IRE and of the light pathway. The Cartesian components of the electric field are shown along the *x*, *y*, and *z* axes. Two possible planes of polarization of the incident light are indicated by $E_{||}$ (polarization parallel to the incidence plane) and $E_{\perp}$ (polarization perpendicular to the incidence plane). The incident beam makes an angle θ with respect to the normal to the IRE surface. The edges of the IRE are beveled so that the incident beam penetrates the IRE through a surface that is perpendicular to its propagation.

cur in the transmembrane domain of the transporter to ensure such modifications.

Structural characterization of MDR transporters can be achieved through 3D crystallization, but membrane proteins are often difficult to overexpress and purify in the quantities required for such trials. Moreover, membrane proteins are notoriously recalcitrant to crystallization, and most successful crystallizations have been carried out in detergent, which rigorous evaluation of the transmembrane domains quite difficult.[12−14] To overcome these difficulties, several other experimental approaches have been developed to obtain information on the structure of the membrane-embedded domains of membrane MDR transporters. We examine here and illustrate with a few examples the potentialities of infrared and fluorescence spectroscopy as alternative tools to investigate the structure, orientation, and conformational changes in membrane-embedded domains of MDR transporters [a bacterial (LmrA) and an eukaryotic (MRP) transporter]. These techniques are fast, yield a strong signal with a few micrograms of sample, and allow the characterization when the protein is in its natural lipidic environment. Furthermore, the environment of the molecules can be modulated so that their conformation can be studied as a function of temperature, pressure, or pH, as well as in the presence of specific ligands.

Attenuated Total Reflection−Fourier Transform Infrared (ATR−FTIR) Spectroscopy to Characterize Transmembrane Domains of Multidrug Transporters

Secondary Structure of Transmembrane Domains. LmrA, a *Lactococcus lactis* protein, mediates antibiotics resistance by using the free energy of ATP hydrolysis to transport a broad range of amphiphilic molecules across the lipid membrane. It is a 590 amino acid protein, containing a N-terminal membrane domain, consisting of 6 transmembrane segments, followed by a hydrophilic domain containing the nucleotide-binding site. Histidine-tagged LmrA was overexpressed in *L. lactis* and purified using a Ni^{2+}-NTA-affinity chromatography.[15] LmrA was then reconstituted into liposomes prepared from *L. lactis* and *Escherichia coli* lipids. After the purification and reconstitution procedures, proteoliposomes exhibited ATP-dependent drug transport.[15] Approximately 20 μg of reconstituted LmrA was spread on the internal reflection element (IRE), and the solvent is slowly evaporated under a gentle N_2 flow to form a film as uniform as possible. This widely used method results in thousands of highly aligned multilayers,[16] which allows the orientation of protein secondary structures to be studied from dichroism measurements (see below). The IRE is a trapezoidal plate that is transparent for the IR radiation of interest. A schematic representation of the IRE appears in Figure 2. Above a critical angle θ, which depends on the refraction index of the IRE (*n1)* and of the external medium (*n2*), the light beam is completely reflected when it impinges on the surface of the IRE.[17] More information about the setup and experimental conditions are available in ref 17.

Figure 4 shows a typical spectrum of reconstituted LmrA. In the 1800−1700 cm^{-1} region, the band corresponding to the C═O stretching of the lipids is visible. One of the most important bands in the study of the protein structure is the amide I band, assigned to the $\nu_{C=O}$ of the peptide bond, which is located in the 1700−1600 cm^{-1} region. This band is by far the most sensitive to the secondary structure of the protein.[18] Because the strength of the hydrogen bonds existing within each secondary structure is different, each secondary structure absorbs at different wavelengths within the amide I region. The frequency limits for each secondary structure were first assigned based on the theoretical[19] and experimental data:[20] 1662−1645 cm^{-1} for α helix; 1689−1682 cm^{-1} for β sheet; 1644−1637 cm^{-1} for random; and 1682−1662 cm^{-1} for β turn. These limits have been slightly adjusted to obtain a good agreement between the data obtained by ATR−FTIR and X-ray crystallography.[18]

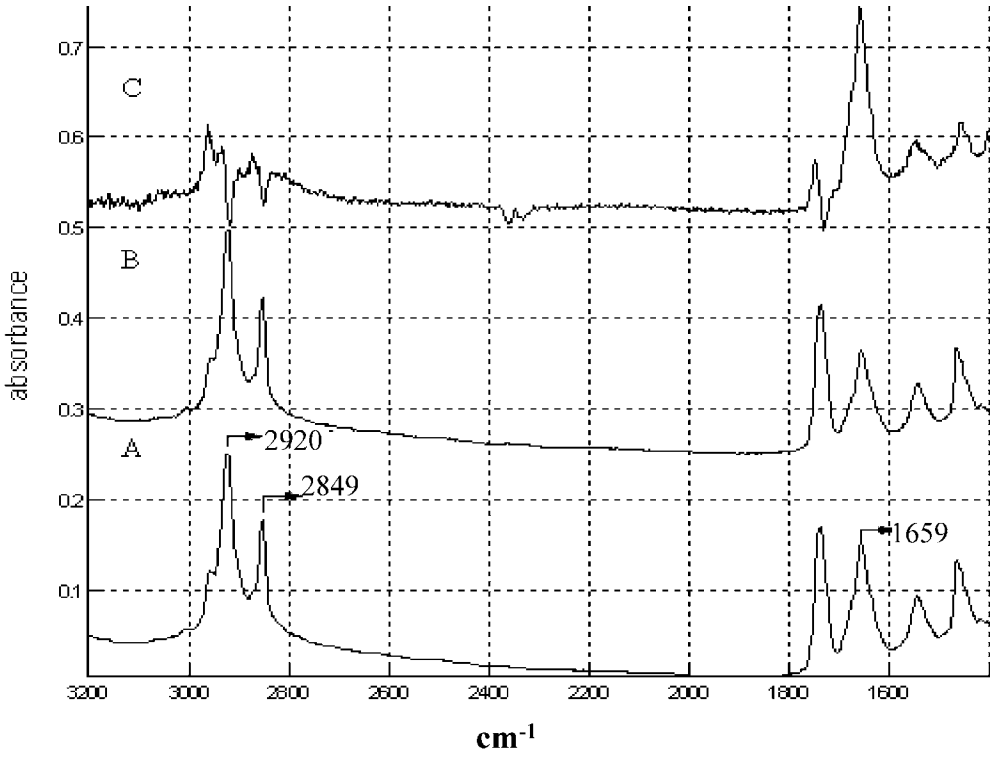

FIGURE 3. Polarized infrared and dichroic spectra of reconstituted LmrA. ATR−FTIR spectra of LmrA recorded with incident light polarized parallel (A) or perpendicular (B) to the incidence plane. Dichroic spectrum (C) is obtained by subtraction of the perpendicularly polarized spectrum from the parallely polarized one as described in the Experimental Procedures. The *y* scale is given in reference to spectrum A, while spectra B and C were offset for clarity. The difference spectra C scale has been increased 4-fold.

From the infrared spectrum, the secondary-structure composition of a protein can be obtained by evaluating the contribution of each secondary structures in the amide I band by a mathematical treatment comprising self-deconvolution and least-squares iterative curve-fitting procedures performed to fit Lorentzian line shapes to the amide I region. The proportion of a particular secondary structure is the sum of the area of all fitted Lorentzian curves.[18] The final result of the curve-fitting procedure applied to the amide I band of LmrA is shown in Figure 4 and enabled us to estimate that the protein contains ~35% α helices, ~24% β sheets, ~28% β turn, and ~13% random.[21] The proportion of α helices is higher than expected from structural predictions:[22] six transmembrane helical segments would correspond to ~20% of the total number of LmrA amino acid residues (590). Therefore, external α helices have to be present. This finding is supported by the high-resolution structure of MsbA, a bacterial homologue of LmrA whose extramembrane domains contain a large number of α helices.[12]

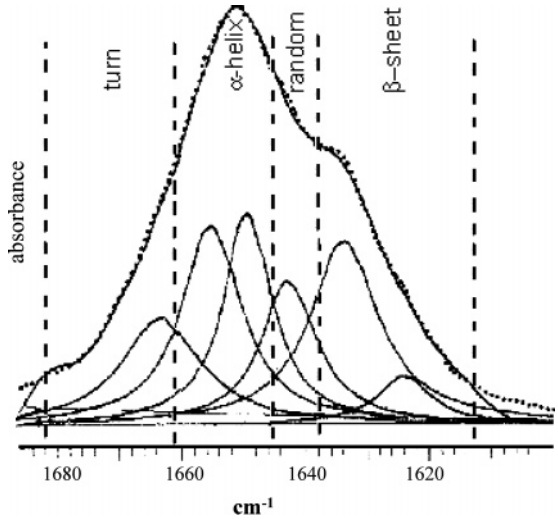

FIGURE 4. Secondary-structure determination using the amide I band of LmrA reconstituted into liposomes. The experimental spectrum appears as a plain line. Each secondary-structure component appears beside this experimental spectrum, with the curve resulting from their addition appearing as a dotted spectrum. Secondary-structure limits of contribution are represented as vertical dotted lines.

Orientation of the Transmembrane Domains. The possibility to gain information on the orientation of different protein secondary structures in a lipidic environment is certainly one of the most exciting possibilities of ATR−FTIR spectroscopy. The method is based on the fact that the infrared light absorption by a chemical bond is maximal if its dipole transition moment is parallel to the electric-field component of the incident light. When the spectral intensity is measured while turning the incident light electric-field orientation with a polarizer, it is possible to obtain information about the orientation of the dipoles. Infrared-polarized spectra of reconstituted LmrA, recorded with the electric-field vector of the IR radiation polarized parallel ($A^{\parallel}$) or perpendicular ($A^{\perp}$) to the incidence plane, appear in Figure 3. The dichroic spectrum is then obtained by subtraction of the perpendicularly polarized spectrum

from the parallely polarized spectrum. As explained above, when an ordered membrane is deposited on the IRE, oriented multilayers of lipids are formed parallel to the IRE. The lipid-associated $\nu_{as}(CH_2)$ and $\nu_s(CH_2)$, which appear at 2920 and 2849 cm^{-1}, respectively, show indeed a negative deviation in the dichroic spectrum, which is in agreement with an orientation of the acyl chains of the lipids essentially perpendicular to the IRE surface in the multilayers build on the IRE[19] (Figure 3). When a membrane protein is inserted in such a membrane, a qualitative analysis of the orientation of its secondary structures with respect to the IRE and consequently to the membrane can be obtained. Figure 3 shows that the amide I band of LmrA displays a strong positive dichroism with a maximum at 1659 cm^{-1}, indicating that some of the helices described above are oriented with respect to the membrane structure. Because the amide I dipole of an α helix can be approximately considered as parallel to its long axis, the positive dichroism detected at 1659 cm^{-1} corresponds to α helices oriented mainly perpendicular to the IRE surface, i.e., perpendicular to the membrane plane.[23] On the contrary, a negative dichroism is indicative of helices oriented parallel with respect to the membrane plane (Figure 5).

Quantitative information about the orientation of secondary structures can be obtained by calculating their dichroic ratio, R^{ATR}, defined as the ratio of the amide I area recorded for the parallel polarization ($A^{||}$) and perpendicular polarization ($A^{\perp}$).

$$R^{ATR} = A^{||}/A^{\perp}$$

In ATR, the dichroic ratio for an isotropic sample, R^{ISO}, is different from unity because of the relative power of the evanescent field for each polarization and is computed based on the area of the lipid ester band (1762−1700 cm^{-1}). The presence in the protein of a fraction x of oriented α helices and $1 - x$ of randomly oriented dipoles results in an experimental dichroic ratio R^{ATR} whose value lays between the value of the oriented helices dichroic ratio R^{α} and of the randomly oriented dipoles R^{ISO}. Determination of R^{α} from x, R^{ISO}, and R^{ATR} can be obtained from[17]

$$R^{\alpha} = A_{\alpha}^{||}/A_{\alpha}^{\perp} = \frac{R^{ATR} - \frac{R^{ATR}+2}{2R^{ISO}+1}(1-x)}{1 - \frac{1}{R^{ISO}}\frac{R^{ATR}+2}{2R^{ISO}+1}(1-x)}$$

In LmrA, analysis of the dichroic spectra demonstrated that $R^{ISO} = 1.5$ and $R^{ATR} = 1.9$. $x = 20\%$ according to the topological model of the protein considering 6 membrane-embedded α helices.[22] The dichroic R^{α} calculated from these values is 2.6. Computing this ratio after the reaction with ligands can be used for instance to detect conformational changes affecting the global orientation of the transmembrane domain (as described in following section). It is also tempting to use these dichroic values to calculate the tilt angles of inserted helices with respect to the lipidic membrane. However, one should have in mind

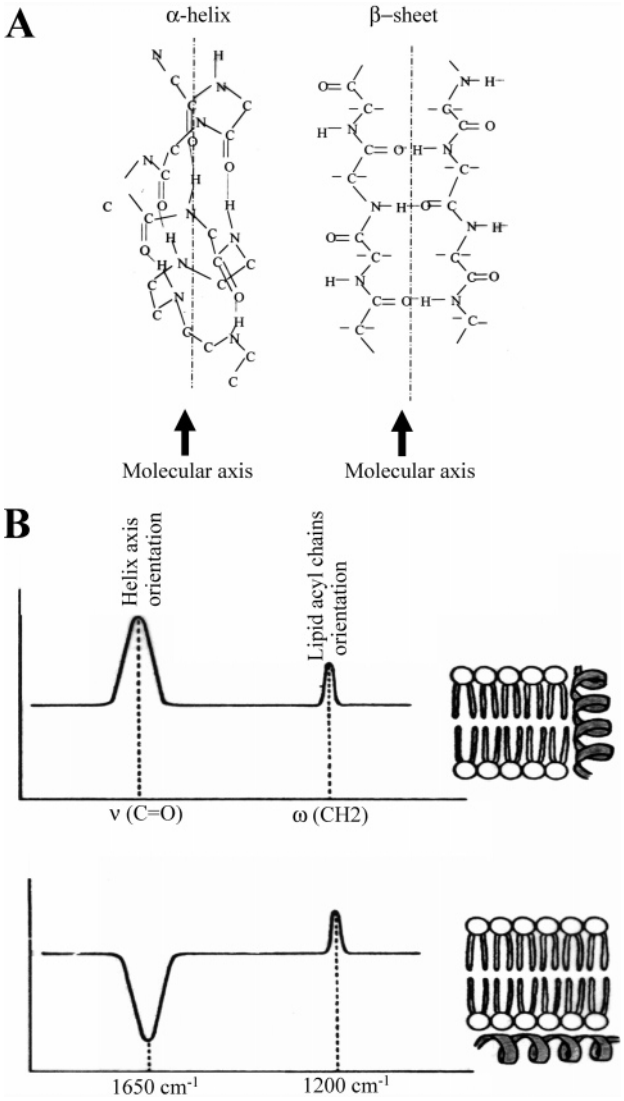

FIGURE 5. Determination of secondary-structure orientation from dichroic spectrum. (A) In an α helix, the molecular axis of the structure is mainly parallel to the amide C═O dipole; in a β sheet, the molecular axis is perpendicular to the amide C═O dipole. (B) Dichroic spectra obtained by subtraction of the ⊥ from the || polarized spectrum. (1) Positive deviation near 1650 cm^{-1} is characteristic of an α-helical structure with the C═O dipole oriented perpendicular with respect to the lipid bilayer. (2) Negative deviation near 1650 cm^{-1} is characteristic of an α-helical structure with a C═O dipole oriented parallel with respect to the lipid bilayer.

that such evaluation is strongly dependent on the order parameter characterizing the lipidic membrane, a parameter that is difficult to estimate especially on the IRE. Moreover, in most cases, membrane proteins contain several transmembrane helices and the tilt angle only represents a mean value.

The dichroic ratio can however be used to prove that the oriented helices detected in LmrA were indeed part of its transmembrane domain. For that purpose, the membrane-embedded domain of LmrA was isolated from the rest of the protein by proteolysis with proteinase K and its spectral characteristics were measured the same way as for the entire protein.[23] With this procedure, we removed the cytoplasmic domain of LmrA, leaving intact

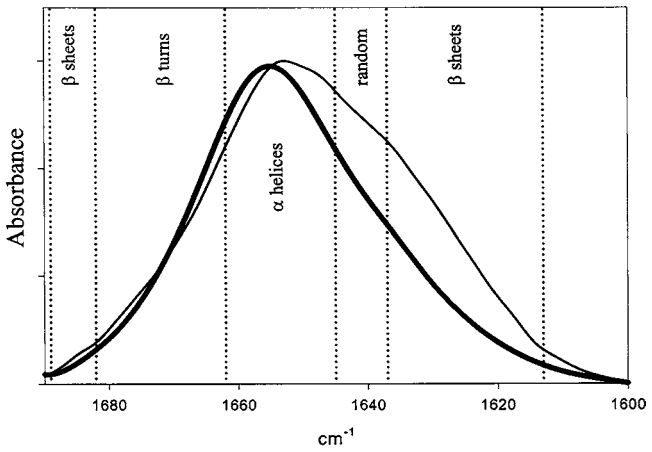

FIGURE 6. ATR−FTIR spectra in the amide I region of intact (solid line) and proteolyzed (dark solid line) LmrA.

the membranous and vesicle interior parts of the protein. Proteolysis indeed indicates a fast cleavage of the protein and the appearance of several peptides. Sequencing of the resulting peptides demonstrated that the 35-kDa peptide encompasses the 6 transmembrane helices and the loop connecting them, while smaller peptides result from internal cleavage of this 35-kDa fragment. This sample was perfectly appropriate to characterize the structural properties of the membrane-embedded domain of LmrA. Indeed, it contained the entire membrane-embedded domain of LmrA without any contribution of the cytoplasmic domain. Infrared spectra of the proteolyzed sample were thus recorded. Analysis of the amide I shape reveals that the α-helix content of the protein increases in the membrane-bound peptides left after proteinase K treatment to 65% in comparison with the 35% in the entire protein, demonstrating that the transmembrane domain of LmrA is essentially made of α helices[23] (Figure 6). The amide I band of the transmembrane region displays a strong positive deviation in the α-helical region of absorption, as observed for the entire protein (data not shown). From $R^{ISO} = 1.5$, $R^{ATR} = 2.4$, and $x = 65\%$, it results that $R^{\alpha} = 2.7$ after proteolysis. This value is almost identical to the one previously detected for the entire protein and indicates that all of the dichroism present in the entire protein can be entirely explained by the dichroim associated to the membrane-embedded helices. Consequently, this analysis demonstrated that the transmembrane domain of LmrA is made of transmembrane helices, which can be detected by using polarized IR spectroscopy.[23]

Conformational Changes in the Transmembrane Domains. The reconstituted protein is spread on the IRE, and oriented multilayers are formed as explained above. The IRE is then placed in an ATR holder for the liquid sample with an inlet and an outlet. H/D exchange is obtained by flushing the sample with nitrogen gas saturated with D_2O by bubbling through a series of four vials containing D_2O. For freely accessible H, the rate of exchange depends only on H exchange chemistry and strongly depends on the pH. In large proteins, secondary structures stabilized by H bonds are resistant to exchange to an extent that depends on their stability. Burial of peptide groups in the hydrophobic regions of a folded protein also prevents the access of the solvent required for H/D exchange. Consequently, evaluating the H/D exchange kinetics of the amide H allows for characterization of the stability and accessibility toward the aqueous phase of the secondary structure that contains the N−H bond. Measuring this H/D exchange in different conditions, such as the presence of ligands, can thus be used to monitor conformational changes affecting these two parameters. In an entire protein, amide H exchange kinetics is followed by monitoring the amide II absorption peak, which is characteristic of δ(N−H) as a function of the time of exposure to D_2O-saturated N_2. During H/D exchange, the area of this band decreases proportionally to the number of H exchanged by D and thus provides a sensitive measurement of the protein stability and accessibility toward the solvent. This method has been extensively used to detect global restructuring during the transport cycle of MDR proteins such as Pgp,[24,25] LmrA,[21,26] and MRP1.[27,28] To monitor exclusively the conformational changes occurring in the membrane-embedded domain of LmrA, the entire spectrum cannot be used because it shows the contribution of the large cytoplasmic domain of the multidrug transporters (Figure 1). On the contrary, the dichroic spectrum of LmrA arises from the oriented helices embedded in the membrane. The amide II band can unfortunately not be used to monitor the H/D exchange kinetics in the dichroic spectrum because it displays low dichroism. During H/D exchange, the C═O vibration is also influenced by the H/D exchange and the amide I band experiences shifts, which are also proportional to H exchanged by D. This suggests that the shift of the amide I dichroic spectrum can provide us with the necessary tool to evaluate specifically the exchange rate of the transmembrane helices: during the time of exposure to D_2O, the shift of the dichroic amide I maximum is correlated to the stability and accessibility toward the external medium of the α helices.

A series of polarized spectra were recorded in the course of the exchange process. Dichroic spectra were generated as explained above. To monitor whether the transmembrane domain of LmrA undergoes conformational changes during its catalytic cycle, we monitored the dichroic amide I shift in the presence of different substrates,[28] the cytotoxic anthracycline daunorubicin (DNR), which is a substrate for LmrA, MgATPγS, and MgATP. The two nucleotides allow for discrimination between the effect of nucleotide binding and hydrolysis on the structure of the protein. A time series of dichroic spectra appears in Figure 7. For the protein alone, the amide I shifts from ∼1658 to ∼1652 cm^{-1} in 10 min. The amide I shift is different in the presence of substrates: it shifts to ∼1653 cm^{-1} in the presence of DNR and to ∼1649 cm^{-1} when ATP is hydrolyzed (Figure 8).

These changes in the shift are due to conformational changes occurring in the transmembrane domain and indicate that the transmembrane helical segments of LmrA are flexible.[29] In particular, the membrane-embedded region undergoes long-range dynamical changes mediated

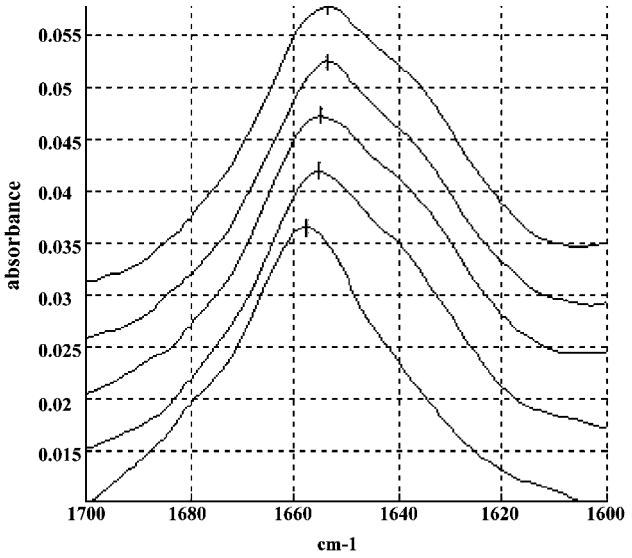

FIGURE 7. Dichroic spectra of LmrA during the H/D exchange kinetics. The spectrum at the bottom has been recorded before deuteration. The other spectra have been recorded at intermediate times (4, 11, 18, and 42 min, respectively).

by the ATP hydrolysis, in addition to local restructuring mediated by drug binding. These restructurings affect the accessibility toward the solvent and/or stability of the membrane-embedded domain, two prerequisites for drug release in the extracellular medium after binding from the lipid bilayer. The R^{α} was determined after addition of ligands as described for the protein alone. No changes were detected, meaning that the restructuring of the membrane-embedded domain during the transport cycle does not modify the global tilt of the transmembrane helices. Consequently, the reorganization most likely results from rotation and/or translation of the α helices within the membrane.

Tryptophan Fluorescence Quenching by Membrane-Embedded Attenuators to Investigate the Conformational Changes of the Transmembrane Domains

Conformational changes in MDR transporters can be measured by monitoring their intrinsic fluorescence because of the presence of Trp residues in their sequence. In particular, Trp fluorescence quenching experiments with aqueous quenchers allowed us to analyze restructurings occurring in the cytosolic domains during the transport process.[30,31] Trp fluorescence quenching can also be used to characterize conformational changes occurring specifically in the membrane-embedded domain of these multidrug transporters. We illustrate this possibility for MRP1. This membrane protein confers resistance to a wide range of chemotherapeutic drugs by rejecting them out of the cells. MRP1 is constituted of two NBDs (NBD1 and NBD2) and two transmembrane domains, each spanning the membrane 6 times. The role of an additional membrane domain of five-spanning domains is not understood. MRP1 contains 30 Trp residues: 10 are located in transmembrane helices as expected from hydropathy analysis[32] (Figure 9). Fluorescence of these Trp residues can be quenched by membranous quenchers and provides a way to detect changes in the Trp locations and hence conformational changes in the presence of substrates. To obtain such data, the protein was overexpressed in BHK-21 cells and purified by Ni^{2+}-NTA-affinity chromatography.[33] The protein was then reconstituted into asolectine vesicles,[27] in which different amounts of brominated phosphatidylcholines (BrPC) were incorporated.[34] These lipids act as quenchers of tryptophan fluorescence and, compared to other membrane-embedded probes such as nitroxide spin-labeled phospholipids or free fatty acids, either spin-labeled or brominated, are less membrane perturbing.[35,36] MRP1 indeed conserves its transport activity in such an environment.

Bromine atoms are attached along the acyl chain at two different positions, namely, the carbon 6 and 7, and quench Trp residues located in close proximity.[34,36] The quenching data were analyzed according to the Stern−Volmer equation for collisional quenching by plotting F/F_0 versus the BrPC concentration.[34] Stern−Volmer plots show deviation from linearity as expected if a proportion of the Trp were inaccessible to the quenchers. In MRP, 20 Trp of 30 are indeed located outside the membrane and consequently inaccessible to the membrane-embedded brominated lipids.[32] Binding of gluthatione (GSH) was studied because it plays a key role in MRP1-mediated drug resistance.[37] Two drugs of the anthracycline family, namely, doxorubicine (DOX) and a doxorubicine analogue, 3′-(3′-methoxymorpholino) doxorubicine (FCE), were also used, in addition to MgAMP−PNP and MgATP nucleotides. As in the LmrA study, these two nucleotides allow us to discriminate between the effect of nucleotide binding or hydrolysis on the structure of the transporter. DOX, a widely clinically used anticancer agent, is actively extruded from cells overexpressing MRP1.[31] In contrast, FCE accumulates identically in drug-sensitive and -resistant cells overexpressing MRP1, demonstrating that it is not transported by MRP1.[31] This permitted us to investigate potential differences in the structure of MRP1 in the presence of transported or nontransported drugs.

Whereas drugs binding did not modify the overall quenching of the Trp located in the membrane (Figure 10A), addition of GSH alone (Figure 10A) or simultaneously with drugs (Figure 10B) affects the structure of the membrane-embedded domains. Addition of nucleotides with drugs and GSH results in two distinct situations, depending on the nature of the drug. Addition of MgAMP−PNP in the presence of DOX and GSH decreased Trp fluorescence quenching (Figure 10C). Replacement of MgAMP−PNP with MgATP induced an additional decrease of Trp fluorescence quenching (Figure 10C). In contrast, when nucleotides were added in the presence of FCE and GSH, no changes in the fluorescence quenching were detected (Figure 10D).

This study provides evidence that the transmembrane segments of MRP1 are not immobile during the transport cycle because different conformational changes were

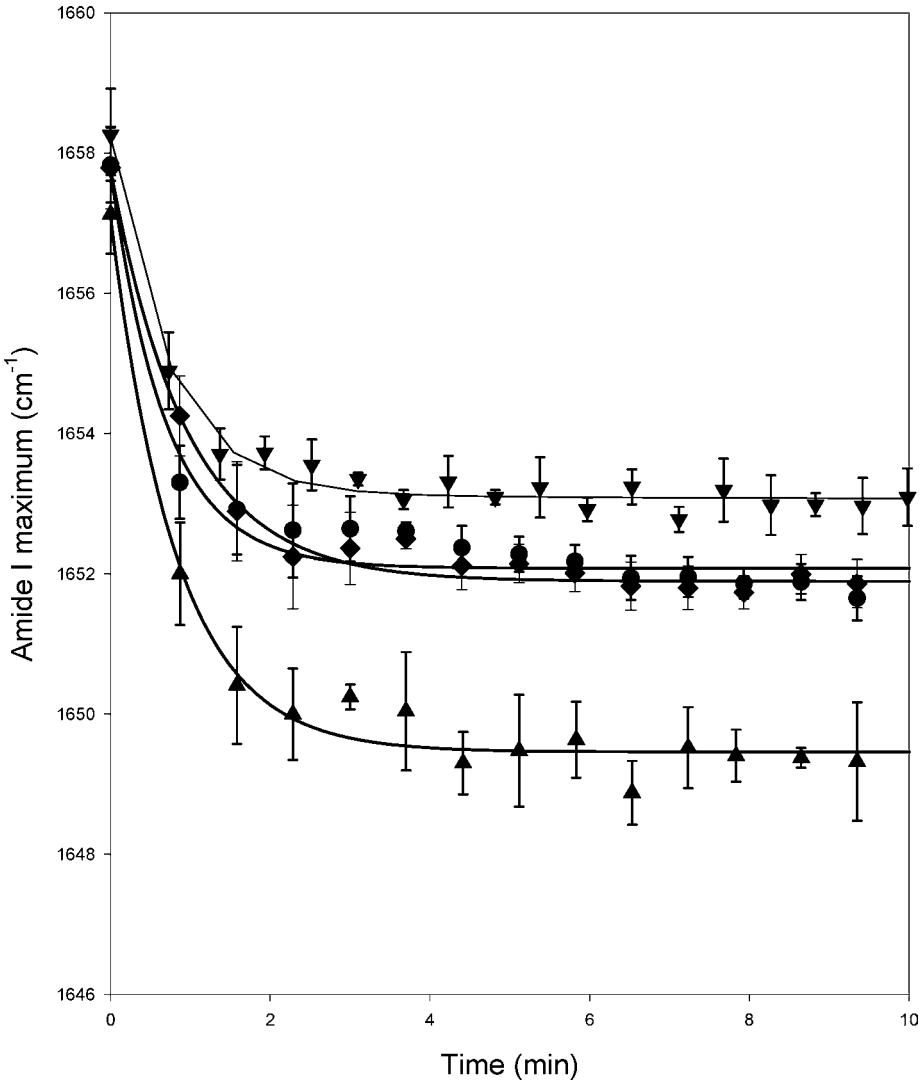

FIGURE 8. Evolution of the amide I maximum of the LmrA dichroic spectrum as a function of the deuteration time. (●) no substrate added, (▼) DNR, (◆) MgATPγS and DNR, and (▲) MgATP and DNR.

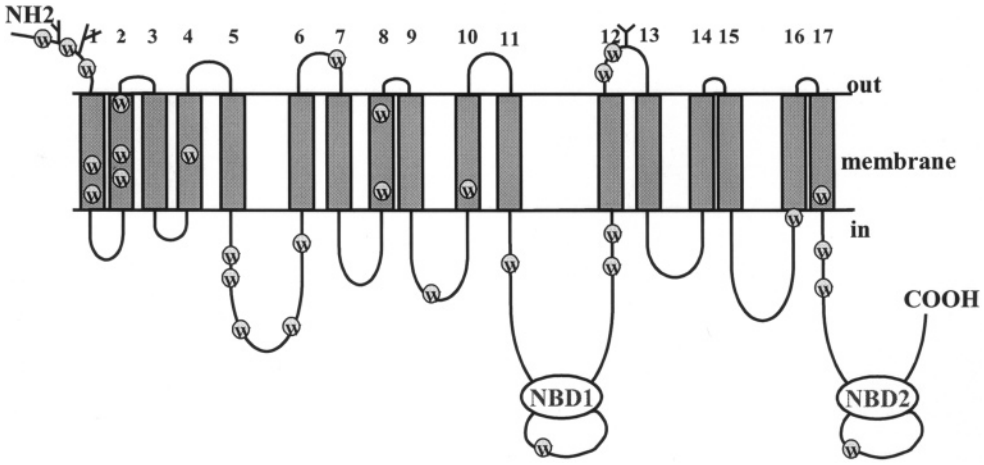

FIGURE 9. Predicted topology of MRP1. Transmembrane helices are depicted as gray rectangles. Trp residues appear as circles. Out and in refer to the extra- and intracellular medium, respectively.

detected in the presence of the substrates. GSH binding reorganizes the transmembrane helices as illustrated by an increase of Trp exposure to the hydrophobic quencher. Conformational changes in the transmembrane domains mediated by ATP binding and hydrolysis occur when transported substrates, like DOX and GSH, are bound to

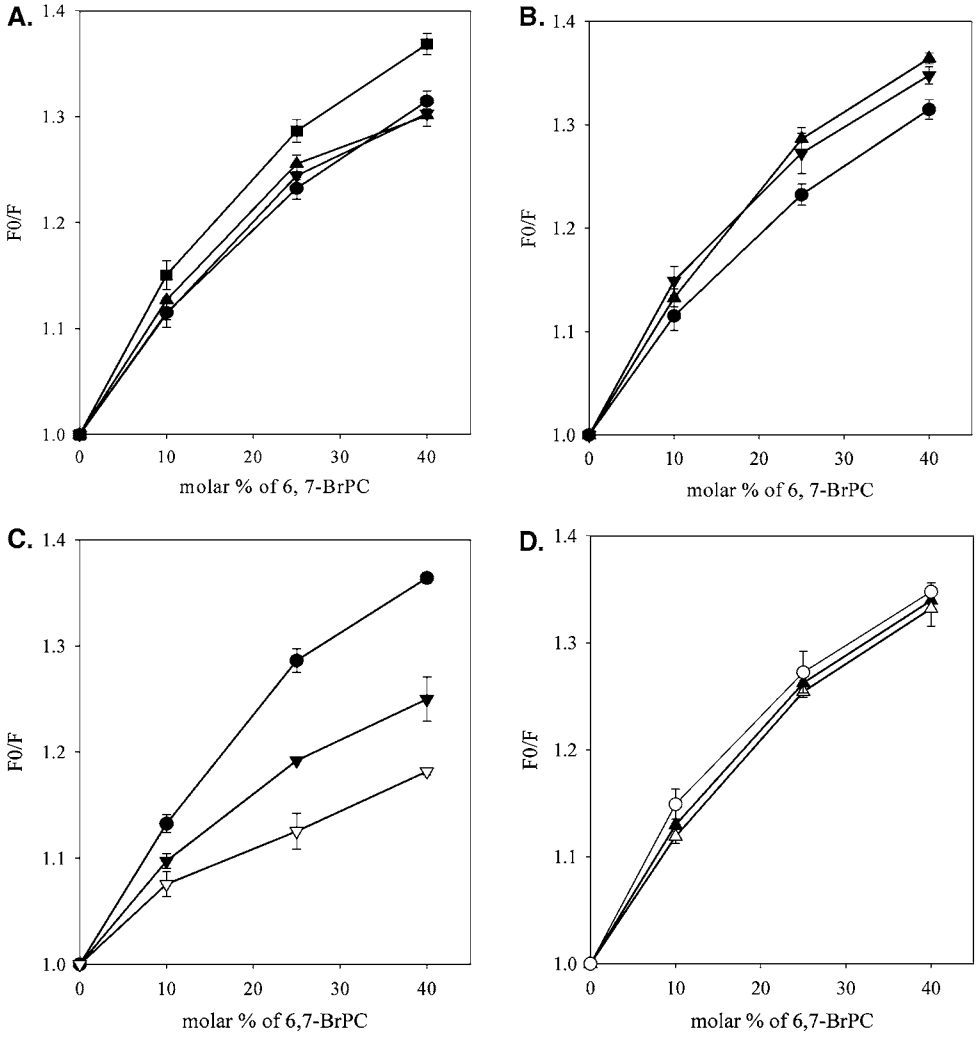

FIGURE 10. Tryptophan fluorescence quenching by 6,7-BrPC incorporated at 10, 25, and 40% (mol/mol) in asolectin vesicles. (A) (●) no ligand added, (■) GSH, (▲) DOX, and (▼) FCE. (B) (●) no ligand added, (▼) FCE and GSH, and (▲) DOX and GSH. (C) (●) DOX and GSH, (▼) MgAMP−PNP, GSH, and DOX, and (▽) MgATP, GSH, and DOX. (D) (○) FCE and GSH, (▲) MgAMP−PNP, GSH, and FCE, and (△) MgATP, GSH, and FCE. F is the measured fluorescence intensity, and F_0 is the initial fluorescence intensity in the absence of 6,7-BrPC.

the protein. A first conformational change is induced by ATP binding and results in a repacking of the transmembrane helices that decreases the exposure of Trp residues to the lipid phase. However, this reorganization is much more drastic after ATP hydrolysis. The situation is quite different when a nontransported drug, like FCE, occupies the binding site. FCE binding blocks the restructuring of transmembrane helices induced by ATP binding and hydrolysis. Interestingly, analysis of the dichroic spectra as performed in the case of LmrA had shown that the tilt angle of the transmembrane helices is not modified when ATP is bound or hydrolyzed in the presence of GSH and DOX.[27] This means that the conformational changes occurring in the transmembrane domain during the transport process are due to rotation and/or translation of the membrane-embedded helices. On the contrary, during ATP hydrolysis in the presence of GSH and FCE, the R^{ATR} and thus the tilt angle of the transmembrane helices are modified. In the presence of a nontransported drug, the membrane-embedded domain containing the drug-binding site is thus modified in an "unusual way" that may inhibit the passage of the drug from its high-affinity-binding site to its low-affinity-binding site. This would explain why FCE remains bound to the protein.[31]

General Conclusions and Perspectives

Infrared and fluorescence spectroscopy offer the opportunity to characterize the secondary structure, orientation, and conformational changes of membrane proteins in their membrane environment and without requiring a large amount of material. These techniques offer notably the opportunity to describe into detail the structural properties of the transmembrane domains of important multidrug transporters such as LmrA and MRP1. Their transmembrane domains are made of α helices oriented mainly perpendicular to the lipidic membrane.[23,28] They are highly flexible and undergo various types of conformational changes during the transport process.[29] First, it appears that ATP binding and hydrolysis in the cytosolic domain of these transporters mediate long-range confor-

mational change affecting the membrane-embedded α helices. In the presence of a transported substrate, this restructuring decreases the stability of the drug-binding domain and increases its accessibility toward the external medium through rotations and/or translations of the transmembrane helices within the membrane but without affecting their global tilt angle. These structural changes are essential prerequisite for the drug release in the external medium. Some cytotoxic agents, such as FCE, are able to modify the influence of the ATPase activity on the structure of the membrane-embedded domain in a way that changes the global tilt angle of the helices. In this new conformation, the crucial changes in substrate affinity and accessibility toward the water phase are probably inhibited and the substrate remains bound to the protein. In such a situation, the transporter protein loses its property to extrude the cytotoxic agent out of the cell. The drug accumulates in the cell and plays its normal cytotoxic activity. The identification of such structural properties mediated by several cytotoxic agents in multidrug transporters and the fact that the techniques used to detect these properties are fast and only require a few micrograms of proteins may provide a useful tool for the screening and design of new effective therapeutic agents in the treatment of cancer and infectious diseases.

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