Structural and Functional Asymmetry of the Nucleotide-binding Domains of P-glycoprotein Investigated by Attenuated Total Reflection Fourier Transform Infrared Spectroscopy*

Received for publication, August 17, 2001, and in revised form, December 7, 2001
Published, JBC Papers in Press, December 10, 2001, DOI 10.1074/jbc.M107928200

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The dynamic changes occurring during the catalytic cycle of MDR3 P-glycoprotein (Pgp) and the role of each nucleotide-binding domain (NBD) in the transport process were investigated using attenuated total reflection Fourier transform infrared spectroscopy. For this purpose, wild-type Pgp and two mutations of homologous residues in each NBD were studied. On the one hand, we demonstrate here that, during its catalytic cycle, Pgp does not undergo secondary structure changes, but only modifications in its stability and accessibility to the external environment. On the other hand, amide H/D exchange kinetics demonstrate that homologous mutations in the two NBDs affect, in a different way, the dynamic properties of Pgp and also the dynamic changes occurring during ATP hydrolysis. These observations led to the conclusion that the NBDs have an asymmetric structure and different functions in the catalytic cycle of Pgp. Our data suggest that the release of drug from the membrane into the extracellular environment is due to decreased stability and/or increased accessibility to the external medium of the membrane-embedded drug-binding site(s). NBD1 would play an important role in this first restructuring of the membrane-embedded domains. NBD2 would be directly implicated in the subsequent restructuring of the membrane-embedded binding sites by which they recover their initial stability and accessibility to the membrane. It is proposed that this restructuring step would allow the binding and transport of another molecule of substrate.

One of the major obstacles to effective chemotherapy of cancer is the multidrug resistance phenomenon (1, 2). This cellular resistance to a wide variety of cytotoxic drugs (Vincap alkaloids, anthracyclines, epipodophyllotoxins, Taxol, etc.) is mainly due to the overexpression in tumor cells of a protein called P-glycoprotein (Pgp) (3). Pgp mediated drug resistance is encoded by one gene in humans (MDR1) and two genes in rodents (mdr1 and mdr3) (2, 4). Pgp uses the energy derived from ATP hydrolysis to transport drugs, out of the cell, against a concentration gradient (5, 6). It is a member of the ATP-binding cassette (ABC) superfamily of proteins (7) and is composed of two homologous halves, each formed by six putative transmembrane helices and one nucleotide-binding domain (NBD) (8). These two homologous halves are separated by a linker region that is thought to allow them to interact properly in the functional molecule (9). The transmembrane domains contain the drug-binding site(s) (10–13) and consequently are believed to form the pathway through which the substrate crosses the membrane. All these topological and functional hypotheses are supported by the high resolution structure of the lipid flippase MsbA from Escherichia coli (14). ATP binding and hydrolysis are mediated by both NBDs, characterized, as in all ABC transporters, by three consensus motifs: the Walker A, Walker B, and ABC signature sequences (15). The high resolution crystal structures obtained for HissP and the NBD of MsbA have clarified the role of many of the residues of these motifs in nucleotide binding and hydrolysis (14, 16).

The Pgp-mediated transport mechanism has been extensively studied, but remains controversial. Each NBD is able to hydrolyze ATP (17, 18), but ATP hydrolysis and drug transport require a cooperation between the two fully functional NBDs (19, 20). UV-induced vanadate (V_i) cleavage experiments demonstrated that only one hydrolysis event can occur at a time (21). All these observations have led to the first catalytic model, proposed by Senior et al. (22), which postulates that Pgp functions by alternating hydrolysis of ATP at the two NBDs. Moreover, to mediate drug binding and subsequent release in the external medium, changes in drug-binding affinity and/or drug-binding site accessibility must occur during the catalytic cycle. The 10-Å resolution structure determined by electron cryomicroscopy of two-dimensional crystals of Pgp (23) together with cross-linking experiments (24) have recently demonstrated the repacking of the transmembrane domains during the transport ATPase cycle. It remains contradictory, however, whether it is ATP binding or the ADP/Pi transition step that mediates the restructuring of the membrane-embedded domains of Pgp during the transport process (23, 25).

One of the unresolved problems in the understanding of the transport mechanism of Pgp is whether both NBDs are equivalent and thus interchangeable or whether each has its own structure and function in the catalytic cycle of the protein. The notion that NBDs in ABC transporters is asymmetric has been demonstrated in a broad variety of systems. Indeed, in the case of eucaryotes, the two NBDs of the cystic fibrosis transmembrane regulator (26), multidrug resistance-associated protein-1 (27, 28), and TAP (transporters associated with antigen
processing) (29) have been shown to have distinguishable activities and functions. The results obtained so far for Pgp remain contradictory. On the basis of photoaffinity labeling experiments using ATP derivatives, Sauna and Ambudkar (30) have proposed that in wild-type (WT) Pgp, the NBDs play the same function. However, applying the same photoaffinity labeling approach to Pgp mutated in the NBDs or to chimeric Pgp, Hrycyna et al. (31) concluded that the two NBDs are essential, but not symmetric. Many other groups have studied the effect of homologous mutations in the two NBDs of Pgp. Depending on the nature of the residue that was replaced, the NBDs appear to be symmetric or not (32, 33). For instance, recent studies have investigated the role of glutamates 552 and 1197 within the NBDs of Pgp encoded by the mdr3 gene (34). Substitution with their uncharged homologs causes a complete loss of drug-stimulated ATPase activity. Indeed, mutants E1197Q and E552Q are able to bind and hydrolyze ATP, but are unable to release Pi and/or ADP after the hydrolysis event, as demonstrated by V$_i$-induced trapping of ADP. However, differences in photolabeling by radiolabeled nucleotides seem to reflect functional differences between the two NBDs (34).

Considering the confusing conclusions in which the techniques used so far have resulted, we decided to use a completely different approach, attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy, to investigate directly differences in photolabeling by radionucleotides seem to reflect the same function. However, applying the same photoaffinity labeling approach to Pgp mutated in the NBDs or to chimeric Pgp, the supernatant was overlaid with a 30 to 5% sucrose linear gradient and centrifuged with an SW 60 rotor. The gradient was fractionated, and the fluorescence intensity. The fluorescence was monitored on an SLM-Aminco 8000 fluorometer using excitation and emission wavelengths of 355 and 457 nm, respectively.

**Experimental Procedures**

**Materials**

ATP, ATP$_7$-S, n-dodecyl β-d-maltoside, and V$_i$ were from Sigma. D$_2$O was from Merck. Bio-Beads SM-2 were from Bio-Rad. Asolectin (soybean phospholipids, Sigma) was purified according to the method described (35) and stored at −20 °C in chloroform. Hoechst 33342 was from Molecular Probes, Inc. Ni$_{2+}$-nitrotetroic acid was from QIAGEN Inc.

**Methods**

**Expression and Purification of WT and Mutant Mouse MDR3 P-glycoproteins**

WT mouse MDR3 Pgp and also Pgp mutants bearing single mutations in the Walker B sequence signature of NBD1 (E552Q) and NBD2 (E1197Q) were constructed in the pPH2 plasmid vector (Invitrogen) and overexpressed in the yeast *Fichia pastoris* as previously described (34, 36). The conditions of expression, solubilization, and purification on Ni$_{2+}$-nitrotetroic acid resin of WT and mutant Pgp from 2-liter cultures induced by methanol were as described (34).

**Reconstitution of WT and Mutant Mouse MDR3 P-glycoproteins**

A dried film of 12.5 mg of asolectin was obtained by evaporation of chloroform under a flow of nitrogen, followed by overnight drying under vacuum. Liposomes were prepared by sonication of the lipid film for 7 min on a Vibra 250W cell sonifier in 6.5 ml of buffer A (10 mM Tris (pH 7.4), 0.5 mM EDTA, 1 mM dithiothreitol, and 75 mM NaCl). n-Dodecyl β-d-maltoside was added to 520 μl of the sonicated lipid suspension to obtain a 1:0.5 (w/w) lipid/detergent ratio. Buffer A was added to obtain a final volume of 750 μl. The mixture was stirred for 20 min at room temperature. 50 μg of purified protein was added to the lipid/detergent mixed micelles, and the volume was adjusted to 1 ml with buffer A. The mixture was stirred for 30 min at room temperature. The detergent was then removed by addition of Bio-Beads SM-2 (three incubations of 2 h under continuous stirring in the presence of 80 mg of Bio-Beads). To demonstrate the association of proteins with lipids, the supernatant collected from the Bio-Beads mixed with an equal volume of 90% sucrose was overlaid with a 30 to 5% sucrose linear gradient and centrifuged overnight at 120,000 × g and 4 °C in a Beckman L7 ultracentrifuge with an SW 60 rotor. The gradient was fractionated, and phospholipid and protein distributions along the gradient were determined by enzymatic colorimetric assay of the phosphatidylcholine and tryptophan fluorescence, respectively. To eliminate the sucrose, the fractions containing the proteoliposomes were washed twice by centrifugation in 3 mM Hepes (pH 7.4) for 2 h at 150,000 × g and then resuspended in ~20 μl of the same buffer.

**ATPase Activity**

ATPase activity was determined by measuring the release of inorganic phosphate from ATP using a colorimetric method adapted from Ref. 37. The liberation of Pi was quantified after 1 h of incubation of the samples (containing 1–2 μg of Pgp reconstituted into liposomes) in the presence of 3 mM MgATP at 37 °C. The contribution of the free hydrolysis of MgATP in the colorimetric assay was subtracted.

**Hoehst 33342 Transport in Proteoliposomes**

25 μl of proteoliposomes was added to 570 μl of buffer A. After 30 s of data acquisition, 35 μl of 100 μM Hoechst 33342 was added. 300 μl of 13 mM MgATP or MgATP-S solution was added after 70 s to reach a final concentration of 3 mM. A control experiment was performed by addition of 300 μl of buffer A to eliminate the effect of dilution on fluorescence intensity. The fluorescence was monitored on an SLM-Aminco 8000 fluorometer using excitation and emission wavelengths of 355 and 457 nm, respectively.

**ATR-FTIR Spectroscopy**

ATR FTIR spectra were recorded at room temperature on a Bruker IFS 55 FTIR spectrophotometer equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector at a nominal resolution of 4 cm$^{-1}$ and recorded every 1 cm$^{-1}$. The spectrophotometer was continuously purged with air. The internal reflection element (ATR) was a germanium plate (50 mm × 20 mm, Harrick EJ2121) with an aperture angle of 45°, yielding 25 internal reflections (38). For each spectrum used for the determination of the secondary structure, 512 scan cycles were averaged. For each kinetic time point, 24 scan cycles were recorded and averaged.

**Sample Preparation**

The sample contained 20 μg of reconstituted WT or mutant Pgp. 0.5 μl of a 6 mM nucleotide solution and/or 3 μl of 10 mM V$_i$ was added to the reconstituted protein. Under these conditions, the molar ratio of Pgp to nucleotides was 1:5. Thin films of oriented multilayers were then obtained by slowly evaporating the sample on one side of the ATR plate in a stream of nitrogen (38, 39). The ATR plate was sealed in a universal sample holder.

**Secondary Structure Analysis**

The sample on the ATR plate was rehydrated by flushing D$_2$O-Saturated N$_2$ for 2 h at room temperature. The determination of the secondary structure was based on the vibrational bands of the protein and particularly the amide I band (1600 to 1700 cm$^{-1}$), which is sensitive to the secondary structure (40). This amide I band, located in a region of the spectrum that is often free of other bands, is composed of 80% pure C=O vibration. The analysis was performed on the amide I region of deuterated samples because the amide H/D exchange allows differentiation of the α-helical secondary structure from the random secondary structure whose absorption band shifts from 1665 to ~1640 cm$^{-1}$ (41). Fourier self-deconvolution was applied to increase the resolution of the spectra in the amide I region. The self-deconvolution was carried out using a lorentzian line shape for the deconvolution and a gaussian line shape for the apodization (40). To avoid the introduction of artifacts due to the self-deconvolution procedure, fitting was performed on the non-deconvoluted spectrum. The proportion of a particular structure is the sum of the area of all the fitted lorentzian bands with their maxima in the frequency region where that structure occurs divided by the total area of amide I. The frequency limits for each structure were first assigned according to theoretical (43) or experimental (44) data: α-helix, 1662 to 1645 cm$^{-1}$; β-sheet, 1689 to 1682 cm$^{-1}$ and 1637 to 1613 cm$^{-1}$; random structure, 1645.4 to 1637 cm$^{-1}$; and β-turn, 1682 to 1662.5 cm$^{-1}$.

**Kinetics of Deuteration**

Films containing 20 μg of reconstituted Pgp in the presence or absence of nucleotides were rehydrated on a germanium plate as described above. Nitrogen was saturated with D$_2$O by bubbling in a series of three vials containing D$_2$O. Before starting the deuterium oxidation, the samples were warmed to test the stability of the measurements and the reproducibility of the area determination. At zero time, the D$_2$O-saturated N$_2$ flux, at a flow rate of 100 ml/min (controlled by a Brooks flow meter), was connected to the sample. The signal from the atmospheric water was subtracted as described by Goormaghtigh and Ruysschaert (45). The area of amide II, characteristic of the δ(N–H) vibration, was obtained by integration between 1596 ...

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and 1502 cm\(^{-1}\). For each spectrum, the area of amide II was divided by the corresponding lipidic \(\nu(C=O)\) area to correct for any change in total intensity of the spectra during the deuteration process (41). This ratio (expressed as a percentage) was plotted versus deuteration time. The 100% value is defined by the amide II/lipid ratio obtained before deuteration. The 0% value corresponds to a zero absorption in the amide II region, observed for full deuteration of the protein.

**RESULTS**

**Reconstitution of P-glycoprotein into Asolectin Liposomes**—

Reconstitution of WT and mutant Pgp was performed by mixing the purified proteins with \(n\)-dodecyl \(\beta\)-\(\omega\)-maltoside-stabilized asolectin vesicles. Detergent was removed by adsorption on polystyrene beads. Proteoliposomes were layered at the bottom of a linear sucrose gradient; and after centrifugation, the gradient was fractionated from the bottom of the tube. Comigration of the proteins and the lipids as a single band demonstrated that proteoliposomes had been formed (data not shown). The proteoliposome population was quite homogeneous, and no aggregates were detectable at the bottom of the gradient. The fractions containing proteoliposomes were pooled and washed twice by centrifugation in 3 mM Hepes (pH 7.4). The proteoliposomes were formed at a 1:22 (w/w) protein/lipid ratio, and 50% of the protein was recovered after these washing steps.

**Drug-stimulated ATPase Activity of Reconstituted Pgp**—The ATPase activity of reconstituted WT Pgp was \(~150 \pm 20\) nmol/min/mg of Pgp in the presence of 3 mM MgATP and was 2-3-fold stimulated by addition of 10 \(\mu\)M verapamil and up to 5-fold stimulated by 50 \(\mu\)M verapamil. This specific activity of murine Pgp is very similar to that previously determined (19, 46). The orientation of Pgp into liposomes was investigated by comparison of its ATPase activity before and after permeabilization of the liposomes in the presence of 2 mM CHAPS (47). This CHAPS concentration has been previously shown to permeabilize the proteoliposomes without modifying the ATPase activity of Pgp (47). We observed that the ATPase activity was not enhanced by addition of 2 mM CHAPS, indicating that Pgp is inserted into the liposomes in an inside-out configuration. As observed previously (34), E552Q and E1197Q showed neither basal ATPase activity nor drug-stimulated ATPase activity.

**ATP-dependent Transport of Hoechst 33342**—Hoechst 33342 has been shown to be a substrate of Pgp (48). It is particularly useful in transport measurements because it is highly fluorescent when bound to lipid membranes, but virtually non-fluorescent in aqueous solution (6). Consequently, the transport function of reconstituted WT Pgp was assayed by measuring the variation of Hoechst 33342 fluorescence as a function of time. In the presence of MgATP, the Hoechst 33342 fluorescence decreased rapidly, whereas no significant change was observed in the presence of the slowly hydrolyzable ATP analog MgATP\(_2\)S (Fig. 1). These observations demonstrate the ATP-dependent transport of Hoechst 33342 by reconstituted Pgp.

**Secondary Structures of WT and Mutant P-glycoproteins**—The spectra of WT Pgp and mutants E552Q and E1197Q reconstituted into lipids were recorded in the absence and presence of nucleotides (MgATP, MgATP\(_2\)S, and MgATP\(_{3}\)V). The IR spectrum of WT Pgp alone in the 1800 to 1400 cm\(^{-1}\) region is shown in Fig. 2. The amide I band, assigned to \(\nu(C=O)\) of the peptide bond, is located in the 1700 to 1600 cm\(^{-1}\) region. The 1570 to 1500 cm\(^{-1}\) and 1450 cm\(^{-1}\) bands represent unexchanged amide II, characteristic of the \(\delta(N-H)\) amide bond, and exchanged amide II, characteristic of the \(\delta(N-D)\) amide bond, respectively. The lipid ester \(\nu(C=O)\) band is located in the 1700–1800 cm\(^{-1}\) region. The secondary structures of WT and mutant Pgp were determined by Fourier deconvolution and curve fitting analysis of the amide I region of a deuterated sample. The secondary structure of WT Pgp was estimated to 46% \(\alpha\)-helices, 18% \(\beta\)-sheets, 8% \(\beta\)-turns, and 28% random structures (Table I). It also clearly appears that the shape of the amide I band (and consequently, the secondary structure of Pgp) was not significantly modified by the point mutations. The secondary structures of WT and mutant Pgp remained unchanged in the presence of nucleotides, demonstrating that Pgp does not undergo secondary structure change during the hydrolysis process.

**Kinetics of Deuteration of WT and Mutant P-glycoproteins**—As amide II (located between 1570 and 1500 cm\(^{-1}\)) arises predominantly from the peptide \(N-H\) bending, the rate of amide H exchanged by amide D is related to the solvent accessibility to the \(N-H\) amide groups and/or to the stability of secondary structure elements. In consequence, it provides sensitive information about the dynamic properties of Pgp. Amide H exchange was followed by monitoring the amide II absorption peak (maximum at 1544 cm\(^{-1}\)) as a function of time of exposure to \(D_2O\)-saturated \(N_2\) (Fig. 2). The percentage of exchange was computed between 0 and 100% as explained under “Experimental Procedures.”

First, the kinetics of deuteration of WT Pgp were monitored in the presence and absence of different nucleotides (MgATP, MgATP\(_2\)S, and MgATP\(_{3}\)V) to observe dynamic changes occurring normally during ATP hydrolysis. MgATP\(_2\)S, the non-hydrolyzable analog of MgATP, was used to distinguish between ATP binding and hydrolysis. To characterize the conformation of WT Pgp after hydrolysis, the protein was stabilized in its ADEPP state by incubation in the presence of \(V_1\) (50). Fig. 3 shows that the exchange rate of WT Pgp was modified by the presence of nucleotides. In the absence of ligand, ~55% of amide H of Pgp exchanged for amide D after 30 min. In the presence of ATP\(_2\)S, the exchange rate measured after 30 min was not modified. However, the H/D exchange occurred faster. In the presence of ATP\(_{3}\)V, the exchange rate drastically increased, as ~75% of amide H was exchanged after 30 min. In the presence of ATP, the exchange rate also increased, but in a less important manner: ~60% of Pgp amide H was exchanged after 30 min.
Second, H/D exchange kinetics were used to investigate the effect of point mutations E552Q and E1197Q on the global dynamic state of Pgp and the dynamic changes occurring during its ATPase activity. As it has been demonstrated that Pgp mutants become unable to release ADP and/or Pi (34), the exchange after hydrolysis was monitored in the presence of ATP only. To further investigate whether mutation in one NBD allows a single turnover in the intact NBD before catalysis comes to a halt and consequently whether the protein can be trapped by V_i in the non-mutant NBD, H/D exchange was monitored in the presence of ATP/V_i. H/D exchanges for WT Pgp (A) and E552Q (B). 

**Fig. 2.** Infrared spectra in the 1800 to 1400 cm\(^{-1}\) region of Pgp actively reconstituted into lipids. Thin films were obtained by slowly evaporating a sample containing 20 μg of Pgp on an attenuated total reflection element. Spectra were recorded as a function of the time (in minutes) of exposure to D\(_2\)O-saturated N\(_2\). Negative times refer to spectra recorded before starting the deuteration procedure. Insets show the decrease in intensity of the amide II band during H/D exchange kinetics of WT Pgp (A) and E552Q (B).

**Table 1.** Secondary structures of WT and mutant Pgps

The percentages were obtained by analysis of the shape of the amide I band according to Goormaghtigh et al. (42).

|         | α-Helix | β-Sheet | β-Turn | Random |
|---------|---------|---------|--------|--------|
| WT      | 46 ± 3  | 18 ± 3  | 8 ± 3  | 28 ± 3 |
| E552Q   | 47 ± 3  | 19 ± 3  | 7 ± 3  | 27 ± 3 |
| E1197Q  | 47 ± 3  | 20 ± 3  | 7 ± 3  | 26 ± 3 |

- Helix
- Sheet
- Turn
- Random

Structure/Function of the Nucleotide-binding Domains of Pgp

Second, H/D exchange kinetics were used to investigate the effect of point mutations E552Q and E1197Q on the global dynamic state of Pgp and the dynamic changes occurring during its ATPase activity. As it has been demonstrated that Pgp mutants become unable to release ADP and/or Pi, the exchange after hydrolysis was monitored in the presence of ATP only. To further investigate whether mutation in one NBD allows a single turnover in the intact NBD before catalysis comes to a halt and consequently whether the protein can be trapped by V_i in the non-mutant NBD, H/D exchange was monitored in the presence of ATP/V_i. H/D exchanges for WT and mutant Pgp in the absence of substrate are shown in Fig. 4A as a function of the deuteration time. About 55% of amide H of Pgp exchanged for amide D after 30 min. This percentage was significantly affected by the homologous mutations, but in a different manner. Indeed, it decreased to ~50% in mutant E1197Q and to ~45% in mutant E552Q. Fig. 4B shows that drastic changes in the exchange rate of E552Q occurred during the hydrolysis process. First, it appears that, after addition of MgATP \(_\text{γS}\), the number of amide H exchanged for amide D after 30 min increased from ~45 to ~60%. In the presence of ATP or ATP/V_i, a drastic increase in the exchange was observed: ~80% of amide H exchanged for amide D after 30 min. On the contrary, the global H/D exchange of E1197Q was only slightly affected by ATP or ATP/V_i (Fig. 4C). ATP/γS increased the percentage of amide H exchanged by amide D after 30 min from ~50 to ~60%. Fig. 4D summarizes the results obtained for the three proteins in the presence of MgATP/γS and shows that the three H/D exchange curves are highly similar.
DISCUSSION

Many recent studies have investigated the catalytic cycle of Pgp mainly using photoaffinity labeling of WT and mutant Pgp (30, 31). All the data are consistent with the model first proposed by Senior et al. (22). This model postulates that Pgp contains two ATP sites that are distinct, but in close communication and alternate catalytically. Furthermore, recent experiments have shown that ATP hydrolysis is responsible for some conformational changes arising in the membrane-embedded domains of Pgp and most probably modifying its affinity for the substrates (23, 24). This change in affinity is thought to allow drug interaction with Pgp and subsequent release in the extracellular medium. Although these works have brought about important findings, the function of each NBD in this transport process remains unclear.

To identify a possible asymmetry in the structure or in the dynamic changes induced by hydrolysis on each NBD, we compared the infrared spectra and H/D exchange kinetics of WT Pgp with those of two mutants, E552Q and E1197Q. These mutants, in which one glutamate of the Walker B sequence is substituted with its uncharged homolog, show a complete loss of their drug-stimulated ATPase activity. They are able to bind and hydrolyze ATP, but are unable to release ADP and/or Pi after the hydrolysis event (34). Through the study presented here, we demonstrate, using ATR-FTIR spectroscopy, that the two NBDs of Pgp are asymmetric in structure and function.

Secondary Structure of Pgp: Structural Effect of Point Mutations in NBDs—The secondary structure determined by ATR-FTIR spectroscopy for WT Pgp encoded by the mouse mdr3 gene is in good agreement with that previously determined by Dong et al. (51) using both CD spectroscopy and prediction from sequence analysis. The high percentage of \(-\alpha\)-helices corresponding to 585 amino acids suggests that, in addition to the proposed 12 membrane-spanning helices, Pgp may contain extramembrane helices. This finding is supported by the high resolution structure of MsaA, a bacterial homolog of Pgp whose extramembrane domains contain a large number of \(\alpha\)-helices (14). The secondary structures of mutants E552Q and E1197Q are similar to that of the WT protein, demonstrating that the substitution of the glutamate residue of the Walker B sequence in NBD1 or NBD2 does not affect the secondary
structure of Pgp. However, mutations E552Q and E1197Q modified the stability and/or accessibility to the external medium of the protein as demonstrated by H/D exchange kinetics (Fig. 4A). The exchange rate after 30 min decreased from −55 to −50% in the E1197Q mutant and to −45% in the E552Q mutant. This observation demonstrates an asymmetric structural effect of homologous mutations in the NBDs and consequently an asymmetric structure of the NBDs themselves. It
was quite unexpected to observe that point mutations affected the dynamics of a large number of amino acids (from −65 to −130). The NBDs themselves are probably not drastically restructured after mutation, as they are still able to bind and hydrolyze ATP (34). Consequently, we would like to suggest that these reorganizations at least partly affect the cytoplasmic loops in close contact with the NBDs. CD measurements have revealed difference in the structures of the two NBDs expressed separately as soluble proteins (52, 53). Here, we provide the first evidence that, in the entire Pgp, the two NBDs have asymmetric structure and/or asymmetric interaction with the other domains of the protein, even before ligand binding.

**Dynamic Changes Induced by the ATPase Activity: Structural Function of Each NBD in the Catalytic Cycle—**Distinct conformational changes have previously been shown to occur during the catalytic cycle of Pgp (54–58). Here, we demonstrate that these intermediate states occurring during the catalytic cycle do not involve changes in the secondary structure of Pgp. Indeed, the secondary structures of WT and mutant Pgp remained unchanged after ATP binding or in the ADP/Pi state. Previous ATR-FTIR experiments had also demonstrated that ADP and verapamil do not affect the secondary structure of Pgp (54). On the contrary, structural changes that modify the stability and/or accessibility to the external environment of the protein occur during the hydrolysis process. In the absence of substrate, the exchange rate of WT Pgp reached a stable value of −55% after 30 min (Fig. 3). The 45% of Pgp that remain unexchanged suggests the presence of tightly folded domains (like the NBDs) in addition to the membrane-embedded domains. A first dynamic change occurred after ATP binding. Indeed, in the presence of ATP/γS, the final exchange rate reached the same level as in the absence of ligand (−55%), demonstrating that nucleotide binding does not modify the global stability or accessibility to the solvent of the protein. However, the exchange kinetics were much faster after ATP binding, which means that the dynamic states before and after ATP binding are distinct. Two-dimensional crystals of Pgp have indeed demonstrated that ATP binding mediates a reorganization of Pgp in its transmembrane domains (23). It was suggested that this structural reorganization affects the affinity of the drug-binding site for the substrate. Our data suggest that this change in affinity is related to a change in the stability of the transmembrane segments without affecting their global accessibility to the external environment. A change in the H/D exchange kinetics was also observed for the two mutants after ATP binding, and Fig. 4D shows that the three proteins reached a similar final H/D exchange rate after ATP binding. This confirms, from a structural point of view, previous experiments of Urbatsch et al. (34), who demonstrated, using photoaffinity labeling with nucleotide derivatives, that ATP binding is not affected in the two mutant proteins. The structure of the three proteins after ATP hydrolysis was then investigated. To stabilize WT Pgp in its ADP/Pi state, the protein was incubated in the presence of ATP and Vγ. Chemical analogy between Pγ and Vγ and x-ray structure analysis of the MgADP-Vγ complex of the *Dictyostelium discoideum* myosin motor domain (59) give strong reasons to think that Vγ in the Pgp-MgADP-Vγ complex occupies the same position as Pγ derived from ATP hydrolysis and that this complex resembles the transition state conformation of the normal reaction pathway. It has been shown that, in the presence of ATP and Vγ, the ATPase activity of Pgp is indeed inhibited and that the protein is stabilized in an ADP/Vγ state after the hydrolysis event (50).

Our study demonstrates that a drastic dynamic change affecting −255 amino acids occurred when the WT protein was trapped in the ADP/Pi state. Indeed, the exchange rate drastically increased from 55 to 75% (Fig. 3). Pgp is supposed to contain 20% of the membrane-embedded amino acids, corresponding to 12 membrane-spanning helices. It is unlikely that the amino acids of WT Pgp that remain unexchanged are only those forming the membrane helices, protected from the exchange by the shielding effect of the membrane. Indeed, on the one hand, other domains like the NBDs, which are well ordered and quite hydrophobic (16, 60, 61), must be a least partly resistant to the exchange. On the other hand, the crystal structure obtained for MshA together with recent experiments on Pgp suggest that tertiary rearrangements of the transmembrane helices mediated by the ATPase activity are implicated in the transport mechanism of these MDR-ABC transporters (14, 23, 24). Consequently, we suggest that the large increase in the exchange of WT Pgp partly corresponds to the restructuring of the membrane-embedded domains in the ADP/Pi state. After this restructuring, the membrane-embedded domains become more accessible to the external medium and/or more flexible, two prerequisites for the release of drug out of the cell. When incubated with ATP, the catalytic cycle of WT Pgp is going on during the data recording. Consequently, the H/D exchange kinetics depend on the structure of four intermediates involved in the catalytic cycle: unbound, ATP-bound, ADP/Pi-bound, ADP-bound Pgp. We have provided evidence that the global exchange rate is not modified by ATP binding, and previous ATR-FTIR measurements have also demonstrated that ADP does not modify the exchange (54). Nevertheless, we observed that the exchange increased from −55% without ligand to −60% in the presence of ATP (Fig. 3). This confirms that the structure of ADP/Pi-trapped Pgp is completely different from those of the three other intermediates.

It has been previously shown that mutants E552Q and E1197Q are unable to release ADP and/or Pγ after ATP hydrolysis and remain blocked in the ADP/Pi state (34); however, it is not known at present in which NBDs the nucleotide is occluded. Consequently, the conformation of these two proteins after hydrolysis was investigated in the presence of ATP, without addition of Vγ. The exchange rate of E552Q increased from −45 to −80% in the presence of ATP (Fig. 4B), demonstrating that a drastic dynamic change affecting −450 amino acids occurred when E552Q was blocked in the ADP/Pi state. As proposed for the WT protein in the presence of ATP/Vγ, we suggest that this restructuring partly affects the membrane domains of Pgp that become highly flexible and accessible to the external environment. On the contrary, the exchange rate of E1197Q trapped in the ADP/Pi state was quite similar to that observed before the interaction with nucleotide (Fig. 4C). However, compared with the ATP-bound state, an increase in stability and/or decrease in accessibility to the external medium occurred in the ADP/Pi state. All these observations demonstrate that, after homologous mutations in the NBDs, the mutants are stabilized in different intermediate states of the catalytic cycle. This suggests that the functions of the NBDs during the two hydrolysis processes required for the transport of one molecule of drug are different. We propose that the decrease in stability and the increase in accessibility to the external medium of the membrane-embedded domains observed in ADP/Pi-trapped E552Q are two prerequisites for the release of drug into the external medium. The function of NBD1 appears to be specifically related to this first step in the transport process of Pgp. On the contrary, the increased stability and decreased accessibility to the external medium observed for ADP/Pi-trapped E1197Q may correspond to the return of the membrane-embedded drug-binding site to its initial state with high affinity for substrate, allowing a new catalytic cycle to occur. During this step of the catalytic cycle of Pgp, the function of NBD2 seems crucial.
Finally, the H/D exchange kinetics of the two mutant proteins were monitored in the presence of ATP/Vi to determine whether the intact NBD of these proteins remains able to hydrolyze ATP and consequently to be trapped in the ADP/Pi state in the presence of Vi. We observed that the kinetics of H/D exchange of E522Q and E1197Q in the presence of ATP/Vi were similar to those measured in the presence of ATP only. This observation suggests that no ADP/Vi state could be trapped in the intact NBD of E552Q or E1197Q. It further demonstrates that the two NBDs can not function independently as catalytic sites in the intact molecule. Vi trapping and photoaffinity labeling experiments have also demonstrated that mutations in one NBD prevent Vi trapping of nucleotide in the intact site.

Translocation Model Proposed for Pgp—A model describing the Pgp-mediated translocation mechanism of substrates across the cell membrane is proposed in Fig. 5. This model is based on biochemical evidence that the drug binds the transporter from the inner leaflet of the bilayer and is translocated across the cell membrane (11, 49). During the transport process, the membrane-binding site alternates between a conformation with high affinity for the substrate, exposed to the inner leaflet of the bilayer (state I in the model), and a conformation with low affinity for the substrate and facing the extracellular medium (state III in the model). We propose that the transition between these two conformations requires two distinct structural changes induced by ATP binding and hydrolysis. In its initial state (I), the membrane-embedded drug-binding site is exposed to the inner leaflet of the membrane and has a high affinity for its substrate. Drug binding occurs in this conformation. Subsequent binding of a first molecule of ATP lowers the affinity of the drug-binding site for its substrate without modifying its accessibility to the extracellular medium (state II). Hydrolysis of this first molecule of ATP exposes the low affinity binding site to the extracellular medium, and drug is released from the transporter (state III). The binding of a second molecule of ATP reorients the low affinity drug-binding site toward the inner leaflet of the membrane (state IV). Subsequent ATP hydrolysis increases its affinity for substrate and allows the binding of another molecule of drug (state I). As explained above, NBD1 seems to play a crucial role in the conformational change occurring during the first hydrolysis event, whereas NBD2 seems to play an important structural function during the second hydrolysis event.

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