Secondary and Tertiary Structure Changes of Reconstituted P-glycoprotein

A FOURIER TRANSFORM ATTENUATED TOTAL REFLECTION INFRARED SPECTROSCOPY ANALYSIS

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The structure of purified P-glycoprotein functionally reconstituted into liposomes was investigated by attenuated total reflection Fourier transform infrared spectroscopy. A quantitative evaluation of the secondary structure and a kinetic of $^2$H/$^2$H exchange of the P-glycoprotein were performed both in the presence and in the absence of MgATP, MgATP-verapamil, and MgADP. This approach was previously shown to be a useful tool to detect secondary structure changes resulting from the interaction between a protein and its specific ligands, as established for the Neurospora crassa H"-ATPase.

$^2$H/$^2$H exchange measurements provided evidence that a large fraction of the P-glycoprotein is poorly accessible to the aqueous medium. Addition of MgATP induced an increased accessibility to the solvent of a population of amino acids, while addition of MgATP-verapamil resulted in a subtraction of a part of the protein from access to the aqueous solvent. No significant changes were observed upon addition of MgADP or verapamil alone. The secondary structure of P-glycoprotein was not affected by addition of ligands. The variations observed in the $^2$H/$^2$H exchange rate when P-glycoprotein interacted with the above ligands therefore represented tertiary structure changes. Fluorescence quenching experiments confirmed that MgATP-induced changes are to be found in the tertiary structure of the enzyme.

P-glycoprotein is a plasma membrane protein member of the "ABC transporters" (ATP-binding cassette) superfamily. This family includes among others the yeast mating pheromone exporter STE6, the mammalian cystic fibrosis transmembrane conductance regulator, the multidrug resistance-associated protein, and the bacterial periplasmic proteins involved in amino acids, sugar, and oligopeptide transport (Hyde et al., 1990; Cole et al., 1992; Higgins et al., 1992; Doige and Ames, 1993).

P-glycoprotein, encoded by the MDR-1 gene in humans, is associated with the resistance frequently developed by cancer cells to a wide variety of cytotoxic drugs used in cancer chemotherapy (Endicott and Ling, 1989; Chan et al., 1991; Pastan and Gottesman, 1991). These hydrophobic drugs, such as Vinca alkaloids, anthracyclines, epipodophyllotoxins, and Taxol, enter cells by passive diffusion through the plasma membrane (Beck and Qian, 1992). P-glycoprotein is thought to mediate multidrug resistance by acting as an ATP-dependent drug pump with a broad specificity. It is able to transport drugs against a concentration gradient to the exterior of the cell (Chin et al., 1993; Gottesman and Pastan, 1993; Sharom et al., 1993; Ruetz and Gros, 1994; Shapiro and Ling, 1995).

According to its amino acid sequence, P-glycoprotein consists of two homologous halves, each including a hydrophobic domain and a hydrophilic region containing the ATP binding domain. Little is known, however, about the structure of P-glycoprotein. Different topologies were derived from experimental studies or from sequence analysis (Chen et al., 1986; Gerlach et al., 1986; Gros et al., 1986; Skach et al., 1993; Zhang and Ling, 1991) but the precise topology of P-glycoprotein needs further study.

P-glycoprotein was recently purified from multidrug-resistant Chinese hamster ovary CH"-B30 cells overexpressing the protein by a combination of anion exchange and immunoaffinity chromatography (Shapiro and Ling, 1994). The purified P-glycoprotein was reconstituted into liposomes where its ATPase activity and ATP-dependent transport properties were retained (Shapiro and Ling, 1994, 1995).

P-glycoprotein displays an ATPase activity in the absence of any known transported molecule and binds MgADP and MgATP, although MgADP is not hydrolyzed (Urbatsch et al., 1995a, 1995b). Hydrolysis of MgATP by P-glycoprotein is stimulated by some chemosensitizers such as verapamil, a calcium channel blocker, and a series of other drugs involved in multidrug resistance (Ambudkar et al., 1992; Sarkadi et al., 1992; Al-Shawi and Senior, 1993; Shapiro and Ling, 1994). Among these molecules, verapamil induces the highest activation (Shapiro and Ling, 1994). Stimulation of ATPase activity suggests that this molecule interacts directly with P-glycoprotein, but the mechanism of stimulation remains unclear. Verapamil, known to reverse drug resistance by inhibiting the efflux of antitumor agents is actively transported by P-glycoprotein (Tsuruo et al., 1981, 1982; Yusa and Tsuruo, 1989). Moreover, a verapamil photoaffinity analogue labels P-glycoprotein (Yusa and Tsuruo, 1989). This evidence supports the hypothesis of a direct binding of verapamil to specific sites of P-glycoprotein.

In this report, we describe the reconstitution of P-glycoprotein in asolectin vesicles and characterize the reconstituted system with respect to protein orientation, ATPase activity, and drug transport. Fourier transform infrared spectroscopy was then used to obtain a quantitative evaluation of the protein secondary structure. The effects of specific ligands, MgATP, MgADP, and verapamil, on the secondary structure of P-glycoprotein were also investigated. Tertiary structure changes were detected by $^2$H/$^2$H exchange rate measurements and fluorescence quenching experiments.
EXPERIMENTAL PROCEDURES

Materials

ATP, ADP, verapamil, octylglucoside, and asolecin were from Sigma. D2O was from Merck. Sephadex G50 was from Pharmacia Biotech Inc. [3H]Daunomycin (3.6 Ci/mmol) was supplied by DuPont NEN.

Methods

Reconstitution

10 μg of P-glycoprotein (0.3 mg/ml in 25 mM Tris-HCl (pH 7.4)). 150 mM NaCl, 1 mM EDTA, 2 mM Chaps1, purified as described (Shapiro and Ling, 1994), was added to a dried film of asolecin and octylglucoside (lipid:octylglucoside ratio of 1:3, w/w). The mixture was centrifuged at 800 × g for 30 min at 4°C. The supernatant was then dialyzed against 0.1 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 2 mM Chaps, and 1 mM DTT for 16 h at 4°C. The resulting protein was recovered.

In order to demonstrate the association of P-glycoprotein with the lipids, the vesicles collected from the Sephadex G50 column were centrifuged at 120,000 × g for 16 h at 4°C in a 29% glycerol solution with a 70% glycerol cushion. Fractones containing lipids were detected by enzymatic colorimetric assay of the phosphatidylcholine (Boehringer Mannheim) and fractions containing protein were detected by trycophan fluorescence. (λex = 280 nm and λem = 330 nm) (Sonneveaux et al., 1994).

A TP Hydrolysis

ATP hydrolysis was measured as described in Shapiro and Ling (1994). Protein determination was performed according to Peterson (1977).

Daunomycin Uptake

A 90-μl aliquot of vesicles containing 5 μg of recompe[nted P-glycoprotein was added to 90 μl of 50 mM Tricine-NaOH (pH 7.4), 4 mM ATP, 8 mM MgCl2, and 4 mM [3H]daunomycin. After an appropriate time at 37°C, the vesicles were centrifuged on a 0.5 × 2-cm column of Sephadex G50 (fine) equilibrated with 50 mM Tricine-NaOH (pH 7.4), 125 mM NaCl, 3 mM ATP, 6 mM MgCl2 to eliminate free daunomycin. 3 ml of scintillation fluid were added to the vesicles collected from the column and 3H)daunomycin uptake was measured by liquid scintillation counting. Association of daunomycin was also measured in the absence of MgATP. The ATP-dependent uptake was calculated by subtracting this uptake from that determined in the presence of ATP.

Infrared Attenuated Total Reflection Spectroscopy

Sample Preparation—Thin films were obtained by slowly evaporating the sample on one side of the ATR plate in a stream of nitrogen. The ATR plate was then sealed in a universal sample holder (Perkin-Elmer) with thin films of the sample. 128 scans were averaged for each measurement. The combination of resolution enhancement methods with a band fitting procedure allows the quantitative assessment of the protein secondary structures (α-helix, β-sheet, and unordered structures). These procedures have been described extensively earlier (Cabiaux et al., 1989; Goormaghtigh et al., 1990a, 1994a; Vigneron et al., 1995).

Kinetics of Deuteration—Films containing 10–20 μg of protein were prepared on a germanium plate as described above. Nitrogen gas was saturated with D2O (by bubbling in a series of five vials containing D2O) at a flow rate of 51 ml/min (controlled by a Brooks flow meter). Bubbling was started at least 1 h before starting the experiment. Before starting the exchange, 10 spectra of the sample dried on the ATR plate were recorded in order to verify the stability of the measure and the reproducibility of the area determination. At zero time, the tubing was connected to the cavity of the film. 12 spectra are recorded and averaged for each kinetic time point. 12 spectra were recorded every 15 s. After the first 2 min, the time interval was increased exponentially. After 16 min, the interval between the scans was large enough to allow the interdigitation of second kinetics. A second sample placed on another ATR setup of the Perkin-Elmer sample shuttle was then analyzed with the same sampling with a 16 min offset by connecting the D2O-saturated N2 flow in series with the first sample. From this time on, our program changed the shuttle position to follow the two kinetics (Goormaghtigh et al., 1994a, 1994b). Typically, one of the samples placed on the shuttle was prepared in the absence of substrate, and the other was prepared in the presence of a substrate as indicated. This procedure allowed us to test the reproducibility of the experiment under identical conditions. Background deuteration kinetics was recorded with the same germanium plate at the same position in the sample shuttle, but in the absence of the sample was subtracted from the kinetics recorded in the presence of the sample. This allowed us to take into account the unavoidable variations in the atmospheric water content inside the spectrophotometer. Indeed, even though the spectrophotometer was purged with dry air for 20 min before starting the experiment, further removal of traces of water vapor took place for several hours, superimposing distinct sharp bands from the water vapor onto the protein spectra (Goormaghtigh and Rouyschaert, 1994).

The subtraction of the background kinetics was improved by adopting the following automated procedure. A subtraction coefficient was first computed as the ratio of the area of the atmospheric water band integrated between 1565 and 1551 cm−1 on the sample spectrum and on the corresponding background spectrum. The areas of amide I, II, and III were obtained by integration between 1702 and 1596, 1596 and 1502, and 1492 and 1412 cm−1, respectively. For each spectrum, the area of amide II was divided by the corresponding lipid νC=O area. This permitted us to take into account small but significant variations of the overall spectral intensity due in part to the presence of D2O, which causes swelling of the sample layer and therefore increases the average distance between the protein sample and the germanium crystal surface. Since the ATR spectrum intensity depends on this distance (Harrick, 1967), this resulted in a loss of a few percent of the band intensity for all measured bands. Undeuterated spectra were recorded before the kinetic experiment as explained above, and 100% deuterated sample values were extrapolated by assuming a value of zero for the amide II surface. The area of amide II (reported to the lipid νC=O area) was finally expressed between 0 and 100% for each kinetic time point.

Fluorescence Quenching Experiments

Iodide quenching experiments were carried out on a SLM Aminco 8000 fluorometer at an excitation wavelength of 280 nm. Increasing amounts of KI were added from a 4 mM stock solution to the proteoliposome suspension (1 ml in water containing 5 μl of recomplemented P-glycoprotein and the various ligands. The KI solution contained 1 mM Na2S2O3 to prevent I and I formation. Fluorescence intensities were read at 333 nm after each addition of quencher. All measurements were carried out at 25°C.

RESULTS

Reconstitution of P-glycoprotein—Purified P-glycoprotein was reconstituted into proteoliposomes by gel filtration chromatography as described under “Experimental Procedures.” Asolecin was chosen as the lipid source since it was previously used for P-glycoprotein reconstitution (Shapiro and Ling, 1995). Two protein:lipid ratios (1:10 and 1:20, w/w) were tested in order to optimize the protein reconstitution. The vesicles collected from the column were centrifuged on a 20% (w/v) glycerol solution with a 70% glycerol cushion to check whether pure protein aggregates were present or whether all the P-glycoprotein recovered in the reconstitution procedure was inserted within the proteoliposome membranes (Fig. 1). This glycerol concentration allows separation of free protein aggregates (at the 20–70% glycerol interface) from proteoliposomes (at the 0–20% glycerol interface).

With a protein:lipid ratio of 1:10 (w/w), two distinct populations of proteoliposomes were detected (fractions 4–6 and fractions 9–12), revealing a nonhomogenous distribution of P-glyco-

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1 The abbreviations used are: Chaps, 3-[3-cholamidopropyl]dimethylammonio-1-propane sulfonate; ATR, attenuated total reflection; FTIR, Fourier transform infrared spectroscopy; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
P-glycoprotein Conformation

FIG. 1. Centrifugation of proteoliposomes reconstituted with P-glycoprotein on a 29% glycerol solution with a 70% glycerol cushion. Fractions containing lipids (●) were detected by an enzymatic colorimetric assay of the phosphatidylcholine and fractions containing proteins (○) were detected by Trp fluorescence (λ<sub>em</sub> = 280 nm, λ<sub>ex</sub> = 330 nm). Panel A, P-glycoprotein reconstituted at a protein:lipid ratio of 1:10 (w/w); panel B, P-glycoprotein reconstituted at a protein:lipid ratio of 1:20 (w/w).

coprotein within the liposomes (Fig. 1, panel A). No free protein aggregates were observed at the 29%-70% glycerol interface. With a protein:lipid ratio of 1:20 (w/w), a single population of proteoliposomes was observed at the 29%-0% glycerol interface (fractions 10–14) and no protein aggregates were detected at the 29%-70% glycerol interface (Fig. 1, panel B). The protein:lipid ratio of 1:20 (w/w) was therefore selected as the more appropriate for P-glycoprotein reconstitution into proteoliposomes.

Drug-stimulated ATPase Activity of Reconstituted P-glycoprotein—It was shown previously that verapamil and a variety of anticancer agents stimulate the ATPase activity of P-glycoprotein, with maximal stimulation occurring in the presence of verapamil (Sarkadi et al., 1992; Al-Shawi and Senior, 1993; Shapiro and Ling, 1994). The effect of verapamil on the ATPase activity was therefore examined. Stimulation of the ATPase activity by verapamil was obtained when P-glycoprotein was reconstituted into proteoliposomes. The ATPase activity of the reconstituted P-glycoprotein (0.03 μmol/min/mg) was 2–3-fold stimulated by 10 μM verapamil (not shown). Addition of 2 mM Chaps to the proteoliposomes did not increase ATP hydrolysis, indicating that P-glycoprotein was inserted into the liposomes in an inside-out configuration with the ATP binding sites exposed to the external aqueous medium. This Chaps concentration was previously shown to permeabilize the proteoliposomes without inhibition of the P-glycoprotein ATPase activity (Shapiro et al., 1995; Sharom et al., 1993).

ATP-dependent Transport of Daunomycin in Reconstituted Proteoliposomes—The transport function of the reconstituted P-glycoprotein was assayed by measuring ATP-dependent accumulation of [3H]daunomycin in the proteoliposomes. Proteoliposomes containing 5 μg of P-glycoprotein were incubated with 2 μM [3H]daunomycin and the uptake of daunomycin into the vesicles was determined both in the presence and in the absence of 3 mM MgATP (Fig. 2). Free drug was eliminated by gel filtration on Sephadex G50. In the absence of MgATP, the uptake measured likely represents passive diffusion of the molecule into the vesicles and partitioning into the membrane bilayer, as well as binding to P-glycoprotein. The difference between daunomycin accumulation in the presence and in the absence of MgATP was therefore considered to represent specific ATP-dependent transport. After 10 min, proteoliposomes treated with MgATP accumulated twice as much daunomycin as proteoliposomes without MgATP (Fig. 2).

Secondary Structure Analysis—The secondary structure of the reconstituted P-glycoprotein was investigated by attenuated total reflection infrared spectroscopy (ATR-FTIR). This method has been shown to be useful for the determination of membrane protein structure (Goormaghtigh et al., 1990a; 1990b; 1993; 1994a; 1994b; Goormaghtigh and Ruysschaert, 1990; Vigneron et al., 1995). Spectra were recorded in the absence and presence of MgATP-verapamil, MgATP, and MgADP. The IR spectra in the 1800–1400 cm<sup>−1</sup> region are shown in Fig. 3. The amide I band, assigned to the ν(C=O) of the peptide bond, is located in the 1700–1800 cm<sup>−1</sup> region. Its maximum at 1654 cm<sup>−1</sup> is associated with a relatively high helicoidal content (Goormaghtigh et al., 1994a). The 1500–1570 cm<sup>−1</sup> and the 1450 cm<sup>−1</sup> bands reflect, respectively, unexchanged and exchanged amide II, characteristic of the δ(N–H) and δ(N–D) amide bond. The lipidic ester ν(C=O) band is located in the 1700–1800 cm<sup>−1</sup> region.

In order to investigate whether modifications of the secondary structure occurred upon binding of the substrates, the spectra of P-glycoprotein recorded after addition of MgATP, MgADP, or MgATP-verapamil were subtracted from the spectrum of P-glycoprotein with no substrate added (Fig. 4). Sub-
tractions were carried out after rescaling all the spectra to the same amide I area. Only minor deviations from the base line were observed in the difference spectra, indicating similar secondary structure contents.

A quantitative evaluation of the secondary structures was obtained by a Fourier deconvolution and a curve-fitting analysis of the amide I region, which is the most sensitive to the secondary structure of proteins (Goormaghtigh et al., 1990a) (data not shown). The secondary structure percentages obtained are summarized in Table I. No significant difference in the protein secondary structure can be detected upon binding of the different molecules to P-glycoprotein (Table I, Fig. 4).

Kinetics of Deuteration—Kinetics of ²H/H exchange of the reconstituted P-glycoprotein was performed in the absence and presence of verapamil-MgATP, MgATP, and MgADP in order to check whether, upon binding of these molecules, the protein undergoes tertiary structure modifications. The infrared spectroscopy methodology allowed us to measure the kinetics of deuteration by recording a series of spectra as a function of the time of exposure to D₂O (Fig. 5). Ten spectra of the protein were recorded before starting the exchange to verify the reproducibility of the measurement. A D₂O-saturated N₂ flow was then flushed in the sample chamber and infrared spectra were automatically recorded at increasing time intervals as the exchange proceeded.

Upon deuteration, the amide II band associated with the δ(N–H) shifts from the 1500–1580 cm⁻¹ region to the 1460 cm⁻¹ region associated with the δ(N–D). The rate of exchange was estimated from the ratio of the amide II area to the corresponding lipidic ν(C=O) area to take into account variations of the ATR spectra intensity due to the swelling of the sample film upon hydration. The decrease of the amide II area, computed between 0 and 100% (see “Experimental Procedures”), is reported as a function of the deuteration time in Fig. 6. As shown in Fig. 6, about 50% of the P-glycoprotein is poorly accessible to the aqueous medium in the absence of ligands. This percentage is not significantly affected upon binding of MgADP (P-glycoprotein/MgADP molar ratio = 1:56). Addition of MgATP to P-glycoprotein (P-glycoprotein/MgATP molar ratio = 1:56) clearly results in an increase of the protein exchange rate. This increase is observed as soon as the exchange begins (as shown in Fig. 6B), and after 2 h of deuteration, the fraction of exchanged amino acids is increased from 50% (P-glycoprotein free of ligands) to 56% (P-glycoprotein with MgATP) (Fig. 6A). P-glycoprotein shows, on the other hand, a significant reduction of its exchange rate upon addition of MgATP-verapamil (P-glycoprotein/verapamil molar ratio = 1:1). The proportion of exchanged amino acids is reduced from 50% (no...
ligands) and 56% (MgATP) to 46% (MgATP-verapamil) (Fig. 6). Verapamil alone has no effect on the $^2$H/$^1$H exchange kinetics of P-glycoprotein (data not shown).

Hydrogen atoms with different exchange rates are implicated in the exchange process. Since the $^2$H/$^1$H exchange is a first order reaction, the exchange curve is expected to display a multiexponential decay corresponding to the different groups of amide protons characterized by a common period $T_i$.

$$H(t) = \sum a_i \exp(-t/T_i) \quad \text{(Eq. 1)}$$

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. Three exponentials characterized by their period $T_i (i = 1–3)$ and by their proportion $a_i$ of amide groups were chosen. A nonlinear fitting of all the experimental curves without constraints on the periods $T_i$ and on the $a_i$ yields three periods $T_1$, $T_2$, and $T_3$. Since these periods $T_1$, $T_2$, and $T_3$, were similar for all the kinetic curves analyzed, a second fitting was performed for each curve, setting the $T_i$ to their average value ($T_1 = 1$, $T_2 = 15$, and $T_3 = 2000$ min) in order to compare the proportion $a_i$ of each amide group for the different experimental curves. The result is shown in Table II.

Addition of MgATP resulted in an increased accessibility to the solvent of a population of slowly exchanging amino acids (76 out of 676 amino acids). These slowly exchanging amide protons ($T_3 = 2000$ min) are essentially changed into fast ($T_1 = 1$ min) and intermediate ($T_2 = 15$ min) exchanging species. Conversely, addition of MgATP-verapamil resulted in protection of a population of fast exchanging amino acids (102 out of 306), thereby confirming a direct interaction between P-glycoprotein and verapamil and demonstrating a verapamil-induced
A major conformational change in the enzyme. Minor changes in amino acid accessibility were observed upon addition of MgADP, characterized by a decrease of about 25 amino acid residues from both the slow and fast exchanging population to the intermediate one (Table II) resulting in the crossing over of the exchange curves (Fig. 6B).

**Fluorescence Quenching Measurements**—Eleven tryptophan residues are present in P-glycoprotein. Tryptophan quenching experiments were therefore performed, both in the absence and in the presence of MgATP and MgADP, to further investigate the effects of specific ligands on the enzyme tertiary structure. Verapamil was not used in these experiments by reason of its fluorescence emission at 333 nm. Fig. 7 shows Stern-Volmer plots of the effects of the aqueous quencher $I^{-}$ on fluorescence intensity. Similar profiles of quenching are observed in the absence and in the presence of 3 mM MgADP. The differences observed in the fluorescence intensities are due to quenching of the initial fluorescence ($F_{0}$), measured in the absence of $I^{-}$, upon addition of MgADP or MgATP to the vesicles suspension. This initial quenching does not affect the fluorescence titration performed by addition of increasing amounts of $I^{-}$. In the presence of 3 mM MgATP, the quenching is much more pronounced confirming thereby that, conversely to MgADP, addition of MgATP resulted in an increased accessibility of a significant fraction of P-glycoprotein to the solvent.

**DISCUSSION**

Many studies have been carried out on cells overexpressing P-glycoprotein or on plasma membrane vesicles. However, these systems can not be used to characterize the protein structure because of the presence of other cellular constituents. Shapiro and Ling (1994, 1995) purified P-glycoprotein from multidrug-resistant Chinese hamster ovary cells and showed that this purified P-glycoprotein retained its ATPase activity and drug transport capability when reconstituted into liposomes. We decided therefore to perform a structure analysis on the purified P-glycoprotein reconstituted into liposomes. For this work, we devised a new reconstitution method yielding proteoliposomes with a high protein:lipid ratio. The resulting proteoliposomes exhibited ATP dependent drug transport and verapamil-stimulated ATPase activity. P-glycoprotein was re-

**Table II**

| Substrate            | $a_1$ | $a_2$ | $a_3$ |
|----------------------|-------|-------|-------|
| No ligand            | 24 (306)* | 23 (295) | 53 (676) |
| MgATP                | 28 (257) | 25 (320) | 47 (600) |
| MgATP-verapamil      | 16 (204) | 27 (345) | 57 (727) |
| MgADP                | 22 (281) | 27 (345) | 51 (650) |

*Numbers in parentheses are the number of amino acids involved.

**Fig. 6.** Evolution of the proportion of exchanged amide bonds computed between 0 and 100% as described under “Experimental Procedures,” as a function of the deuteration time. ○, no substrate added; ●, MgADP (P-glycoprotein/MgADP molar ratio = 1:56); ▼, MgATP (P-glycoprotein/MgATP molar ratio = 1:56); ▽, MgATP-verapamil (P-glycoprotein/verapamil molar ratio = 1:1). A, reported between 0 and 120 min; B, reported between 0 and 20 min. The curves presented are the means of three experiments. The error bars represent the standard deviation.
amino acid content of the protein. The presence of a large MgATP suggests that MgATP-induced changes are to be found as a conformational change in P-glycoprotein in the presence of alteration in P-glycoprotein secondary structures (Fig. 4 and Table I). MgATP, MgATP-verapamil, or MgADP does not significantly change membrane-spanning helices. The proportion of protein contains two hydrophobic domains, each consisting of six amino acid residues involved. Comparison of ATR-FTIR experiments carried out on films with data obtained in solution by FTIR or NMR demonstrate the validity of this approach (de Jongh et al., 1995).

\[ F = \frac{F_o}{F} \]

FIG. 7. Stern-Volmer plots of aqueous quenching by \( I^- \) of reconstituted P-glycoprotein. \( F \) is the measured fluorescence intensity and \( F_o \) is the fluorescence intensity in the absence of \( I^- \). ○, no ligand added; ■, 3 mm MgADP; and ▲, 3 mm MgATP.

constituted with an inside-out configuration.

Infrared spectroscopy analysis of the functionally reconstituted P-glycoprotein provided information concerning the secondary and tertiary structure of the protein. The proportions of secondary structures estimated by ATR-FTIR were 32% α-helix, 26% β-sheet, 29% β-turn, and 13% unordered structure. The P-glycoprotein amino acid sequence suggests that the protein contains two hydrophobic domains, each consisting of six membrane-spanning helices. The proportion of α-helices determined experimentally is higher than the one expects just for the 12 putative membrane-spanning α-helices predicted, which represent only 20% of the protein. External α-helices or additional transmembrane helical segments have therefore to be considered. It is likely that the large cytoplasmic domains contains some α-helical structure. Importantly, addition of MgATP, MgATP-verapamil, or MgADP does not significantly alter P-glycoprotein secondary structures (Fig. 4 and Table I). The fact that limited trypsin proteolysis experiments indicated a conformational change in P-glycoprotein in the presence of MgATP suggests that MgATP-induced changes are to be found in the tertiary structure of the enzyme. A similar result was recently obtained on a P-type H^+−ATPase (Goormaghtigh et al., 1994b). While various ligands are known to modify the limited trypsin proteolysis pattern, the secondary structure of the enzyme was not affected (Goormaghtigh et al., 1994b).

When the secondary structure is constant, amide \(^2\)H/H exchange experiments bring information on protein amide accessibility from the solvent and thereby allow detection of tertiary conformational changes and quantification of the number of amino acid residues involved. Comparison of ATR-FTIR experiments carried out on films with data obtained in solution by FTIR or NMR demonstrate the validity of this approach (de Jongh et al., 1995).

\(^2\)H/H exchange measurements provided evidence that a large fraction of the P-glycoprotein is poorly accessible to the aqueous medium. The slowly exchanging amino acids, characterized by a half-decay \( T_{1/2} = 2000 \) min, represent 53% of the total amino acid content of the protein. The presence of a large amide population characterized by a very low exchange rate could be due, in part, to the shielding effect of the membrane on a large number of residues.

Transmembrane domains of several membrane proteins such as glycoporphin or bacteriorhodopsin are inaccessible to the solvent as shown by their slow kinetics of amide \(^2\)H/H exchange (Challou et al., 1994; Vigneron et al., 1995). The slowly exchanging amide protons of P-glycoprotein could therefore be formed by a core of amino acids located within the membrane. However, according to the protein sequence, the predicted transmembrane domains of P-glycoprotein account for only 20% of the total amino acids. A significant proportion of the slowly exchanging amino acids must therefore be located outside the membrane where they likely form highly structured domains.

Interestingly, \(^2\)H/H exchange kinetics revealed major conformational changes upon addition of MgATP (increased accessibility for at least 76 amino acid residues) and MgATP-verapamil (decreased accessibility of at least 102 amino acid residues). However, as shown in Table II and in Fig. 6, the differences observed between MgADP and the control were not as clear, and we did not attempt to obtain any interpretation in terms of conformational change. No conformational change was observed upon addition of verapamil alone (data not shown), demonstrating that the verapamil-induced conformational change requires MgATP.

Assigning the exchange differences to changes in the tertiary structure of the protein, even in the absence of secondary structure change, is not necessarily obvious. Indeed, exchange is likely to occur during transient (partial) unfolding of the secondary structures, and any perturbation of the system which increases the frequency of the transient unfolding event will have a significant impact on the exchange rate. This argument remains valid even if the transient unfolded species represents a very small fraction of the structure when compared to the ordered secondary structure. For instance, denaturing agents such as urea, temperature, or pH will increase the transiently unfolded species concentration, and thereby the \(^2\)H/H exchange rate, even though the secondary and tertiary structures are essentially unchanged. In the present study, because of the specificity of the ligands, no global, unspecific effect is expected. It is clear that, if unspecific effects were to occur, we would expect a similar effect from ADP and ATP, while we find different effects on the \(^2\)H/H exchange proving the specificity of the interaction, necessarily highly localized on the polypeptide chain. Yet, a large fraction of the polypeptide chain (at least 76 amino acid residues upon addition of ATP, see Table II) is affected even though ATP has only two binding sites on the protein and therefore cannot interact directly with 76 amino acid residues. Reconciling the highly localized binding of ATP on the polypeptide chain with its large spread effect on the exchange properties suggests a global tertiary structure change in the protein, but does not rule out a local destabilization of the tertiary structure. However, fluorescence quenching experiments indicated that, upon addition of MgATP, the enzyme adopts a different tertiary structure resulting in a significantly increased solvent accessibility of its tryptophan residues. Such major conformational changes were not observed upon binding of MgADP.

Similarly, a P-type ATPase was recently shown to undergo major tertiary structure changes involving up to 175 out of a total of 920 amino acid residues upon addition of ligands. In contrast to P-glycoprotein, however, binding of MgADP or MgATP-vanadate to the P-type H^+−ATPase resulted in protection of a large population of amino acids (Addison and Scarborough, 1982; Goormaghtigh et al., 1994b). The major differences

\(^2\)M. Zhang, A. B. Shapiro, and J.-T. Zhang, personal communication.
observed between P-glycoprotein and the P-type proton ATPase. Upon addition of ligands, interactions between P-glycoprotein and the molecules transported may be related to the stimulation of the ATPase activity by verapamil and MgATP, consistent with direct stimulation of the ATPase resulting from verapamil binding. This would require tight conformational coupling between the two sites, as suggested by Urbatsch et al. (1995a, 1995b) that ATP hydrolysis by the two nucleotide binding sites of P-glycoprotein may occur by an alternating site mechanism. This would require tight conformational coupling between the two sites and may be related to the stimulation of the ATPase upon addition of verapamil and MgATP.

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