Ligand-mediated Tertiary Structure Changes of Reconstituted P-glycoprotein

A TRYPTOPHAN FLUORESCENCE QUENCHING ANALYSIS*

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Ligand-dependent changes in accessibility of purified P-glycoprotein, functionally reconstituted in liposomes, were investigated by fluorescence measurements. Trp quenching experiments provided evidence that P-glycoprotein adopts different tertiary structures upon binding of drug substrates in the absence and presence of MgATP and its nonhydrolyzable analog, MgATPγS. Five anthracycline derivatives were tested as drug substrates: daunorubicin, 4′-epi-doxorubicin, iododoxorubicin, 4-demethoxy-daunorubicin, and methoxy-morpholino-doxorubicin. Among them, daunorubicin and 4′-epi-doxorubicin have been shown to be rejected outside the multidrug-resistant cells, whereas the other three have been shown to accumulate in multidrug-resistant cells overexpressing P-glycoprotein and therefore retain their cytotoxic activity. A small conformational change was associated with nucleotide binding and amplified after nucleotide hydrolysis. Different conformational states were adopted by P-glycoprotein upon the addition of the anthracycline derivatives in the absence and presence of MgATP or MgATPγS. These conformational changes are shown to be related to the nature of the antitumor agents and more precisely to their capacity to accumulate in resistant cells. These data also suggest that the cytotoxicity of iododoxorubicin and 4′-demethoxy-daunorubicin is related to the fact they are not transported by P-glycoprotein. On the contrary, methoxy-morpholino-doxorubicin cytotoxicity may be explained in terms of its rapid reincorporation into the plasma membrane after being transported by P-glycoprotein.

P-glycoprotein is a 170-kDa plasma membrane protein involved in the multidrug resistance phenomenon responsible for failure of many human cancer chemotherapies (1). A structural prediction based on its sequence predicts two homologous halves, each containing six putative membrane-spanning α helices and a cytoplasmic nucleotide-binding domain (NBD)1 with characteristic Walker motifs A and B. However, experimental studies concerning this proposed topology (2–5) remain controversial. According to its sequence, P-glycoprotein is classified as a member of a large family of membrane transporters known as the ATP-binding cassette superfamily that includes yeast, bacteria, and mammalian transporters (6, 7). P-glycoprotein is proposed to function as an ATP-driven efflux pump, transporting through the plasma membrane an unusually broad but well defined spectrum of structurally unrelated cytotoxic drugs, including the Vinca alkaloids, anthracyclines, epipodophyllotoxins, and taxanes (8–10).

A large body of evidence suggests that the transmembrane domains of the P-glycoprotein participate in the recognition of substrates, whereas the ATP hydrolysis necessary for transport is carried out by both NBD regions with a similar efficiency in an alternating fashion (11–16). The mechanism of coupling ATP hydrolysis at the two cytoplasmic nucleotide-binding sites to drug transport by the intramembrane drug-binding site(s) is likely to involve substantial conformational changes in the P-glycoprotein structure (17). Different tertiary conformational changes have previously been shown to take place upon addition of MgATP and MgATP-verapamil, an actively transported chemosensitizer (18). The comprehension of the mechanism of interaction between the drug-binding site(s) and the NBD domains is essential for understanding how P-glycoprotein transports its substrates. The aim of this study was to further investigate the different conformations adopted by the protein in the presence of nucleotide ligands and drugs.

Purified P-glycoprotein from CHO cells was reconstituted into lipid vesicles with an “inside-out” orientation, exposing its cytoplasmic region, which contains the NBD domains, to the external medium. The resulting proteoliposomes have previously been shown to exhibit both ATP-dependent drug transport and drug-stimulated ATPase activity (18).

Acrylamide quenching of Trp fluorescence was used to monitor ligand-dependent changes in the accessibility of reconstituted P-glycoprotein in the presence of MgATP, MgATPγS, a nonhydrolyzable analog of MgATP, and of five anthracycline antitumor agents (daunorubicin, 4′-epi-doxorubicin, iododoxorubicin, 4′-demethoxy-daunorubicin, and FCE) previously used in pharmacokinetics studies on multidrug-resistant and sensitive K562 cells (19). Three of these agents (iododoxorubicin, 4′-demethoxy-daunorubicin, and FCE) have been shown to accumulate within multidrug-resistant cells overexpressing P-methoxy-morpholino-doxorubicin; Hoechst 33342, 2-[2-(4-ethoxyphenyl)-6-benzimidazolyl]-6-(1-methyl)-4-piperazil-benzimidazole; ATPγS, adenosine 5′-O-(thiotriphosphate); Chaps, 3-[[(3-cholamidopropyl)]dimethylammonio]-1-propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis( hydroxymethyl)ethyl]glycine.

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1 The abbreviations used are: NBD, nucleotide-binding domain; FCE, [2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
glycoprotein and to retain their cytotoxic activity. On the contrary, daunorubicin and 4′-epi-doxorubicin were rejected outside the multidrug-resistant cells. We analyze here how the P-glycoprotein conformational changes are related to the nature of the antitumor agents and, more precisely, to their capacity to accumulate into resistant cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

Daunorubicin, ATP, ATPγS, octylglycoside, and asolectin were from Sigma. [3H]Azidopine (specific activity of 40 Ci/mol) was purchased from Amersham Pharmacia Biotech. 4′-epi-doxorubicin, iododoxorubicin, 4-demethoxy-daunorubicin, and FCE were obtained from Pharmacia-Farmitalia (Milan, Italy). Sephadex G-50 was from Amersham Pharmacia Biotech.

**Methods**

**P-glycoprotein Reconstitution**—8 μg of P-glycoprotein (0.3 mg/ml in 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 2 mM Chaps), purified as described previously (20), was added to a dried film of asolectin (protein:lipid ratio of 1:20 w/w) and octylglycoside (lipid:octylglucoside ratio of 1:3 w/w). The mixture was centrifuged at 500 × g for 30 s on a 0.7 × 2-cm column of Sephadex G-50 (fine) equilibrated with 125 mM NaCl and 50 mM Tricine-NaOH (pH 7.4). The proteoliposomes were collected in about 60 μl, 50% of the protein was recovered.

**ATP Hydrolysis**—ATP hydrolysis was measured as described by Shapiro and Ling (20) in the absence and presence of increasing concentrations (2, 10, and 50 μM) of each anthracycline derivative. Protein determination was performed according to Peterson (21).

**Fluorescence Quenching Experiments**—Acrylamide quenching experiments were carried out on a SLM Aminco 8000 fluorimeter at an excitation wavelength of 295 nm instead of 280 nm to reduce the absorbance by acrylamide. Control experiments established that no emission of the anthracycline derivatives occurred at 340 nm if excited at 295 nm (see Ref. 22 for excitation spectrum, ~390–540 nm, of daunorubicin). Acrylamide aliquots were added from a 3 M solution to the proteoliposome suspension (1 ml in water) containing 8 μg of reconstituted P-glycoprotein and the various ligands. The final concentration was 3 mM for MgATP and MgATPγS and 10 μM for the various anthracycline derivatives. Fluorescence intensities were measured at 340 nm after each addition of quencher. All measurements were carried out at 25 °C. Acrylamide quenching data were subjected to a linear fit up to 80% absorbance by acrylamide. Control experiments established that no static quenching by acrylamide occurred upon addition of the five anthracycline derivatives in darkness at room temperature for 1 h with 0.3 μM [3H] azidopine and the indicated concentrations of anthracycline derivatives. After incubation, the samples were exposed to a UV lamp for 10 min while being kept on ice. Laemmli’s buffer was added, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis and by autoradiography of the dried gel.

**RESULTS**

**Fluorescence Experiments**—Fluorescence experiments were performed to investigate changes in P-glycoprotein structure that occur upon binding of drug and nucleotide substrates. Five anthracycline derivatives, cytotoxic (iododoxorubicin, 4-demethoxy-daunorubicin, and FCE) and noncytotoxic (daunorubicin and 4′-epi-doxorubicin) to resistant cells, were used (Fig. 1). These were added to a final concentration of 10 μM to P-glycoprotein-containing proteoliposomes in the presence and absence of MgATP (3 mM) or MgATPγS (3 mM), a nonhydrolyzable MgATP analog. This analog allows discrimination between the influence of nucleotide binding and nucleotide hydrolysis on P-glycoprotein structure. The exposure of P-glycoprotein Trp residues to the external solvent was subsequently determined by continuous monitoring of P-glycoprotein fluorescence intensity in the presence of increasing concentrations of acrylamide (0–0.1 M), a neutral aqueous quencher.

Fig. 2 shows Stern-Volmer plots of P-glycoprotein Trp quenching by acrylamide in the absence and presence of nucleotides. F is the measured fluorescence intensity, and F0 is the initial fluorescence intensity in the absence of acrylamide. ●, no ligand added; ○, 3 mM MgATP; ▽, 3 mM MgATPγS. The results are the means of three experiments. The error bars represent the standard deviation.

**Fig. 2.** Stern-Volmer plots of P-glycoprotein Trp quenching by acrylamide in the absence and presence of nucleotides. F is the measured fluorescence intensity, and F0 is the initial fluorescence intensity in the absence of acrylamide. ●, no ligand added; ○, 3 mM MgATP; ▽, 3 mM MgATPγS. The results are the means of three experiments. The error bars represent the standard deviation.
binding to P-glycoprotein resulted in a decreased accessibility of the Trp to the aqueous solvent. Upon co-addition of MgATP or MgATPγS and drug substrates, the quenching efficiency was observed to depend on the nature of the drug added (Figs. 4 and 5).

In the presence of drugs noncytotoxic to resistant cells (daunorubicin and 4’-epi-doxorubicin) known to be transported by P-glycoprotein, addition of MgATP resulted in the stabilization of the enzyme in a conformational state intermediate between that observed in the presence of MgATP alone and that observed upon binding of the five drugs (Fig. 4A). Replacement of MgATP by its nonhydrolyzable analog, MgATPγS, had no effect on the levels of quenching observed (Fig. 4B). This indicates that nucleotide binding was necessary and sufficient to modify the accessibility of the protein to the water phase.

Upon addition of drugs cytotoxic to resistant cells (iododoxorubicin, 4’-demethoxy-daunorubicin, and FCE) and MgATP or MgATPγS, two distinct situations were observed (Fig. 5): 1) in the presence of iododoxorubicin and 4’-demethoxy-daunorubicin, no fluorescence quenching was detected even after addition of MgATP or MgATPγS and 2) in the presence of the morpholino derivative (FCE), no fluorescence quenching was detected after addition of MgATPγS, but addition of MgATP led to substantial quenching of the protein fluorescence. In the presence of MgATP, the accessibility of the protein was identical to that observed with transported noncytotoxic molecules. Stern-Volmer constants were calculated in each case (Table I).

**ATPase Activity Measurements in the Presence of Anthracycline Derivatives**—Upon binding of anthracycline derivatives, except for the FCE derivative, P-glycoprotein adopted the same conformations when MgATP was replaced by its nonhydrolyzable analog MgATPγS. Furthermore, MgATP had no influence on P-glycoprotein conformation when iododoxorubicin and 4’-demethoxy-daunorubicin were bound to the protein, suggesting that the nucleotide could not access the ATP-binding sites in this conformation. P-glycoprotein ATPase activity was therefore measured in the absence and presence of each of the

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**Fig. 3.** Stern-Volmer plots of P-glycoprotein Trp quenching by acrylamide upon addition of daunorubicin. Addition of 4’-epi-doxorubicin, iododoxorubicin, 4-demethoxy-daunorubicin, or FCE to P-glycoprotein give identical Stern-Volmer plots (data not shown). F is the measured fluorescence intensity, and F₀ is the initial fluorescence intensity in the absence of acrylamide. ●, no ligand added; □, 10 μM anthracycline derivative. The results are the means of three experiments. The error bars represent the standard deviation.

**Fig. 4.** Stern-Volmer plots of P-glycoprotein Trp quenching by acrylamide upon co-addition of noncytotoxic anthracycline derivatives and MgATP (A) or MgATPγS (B). F is the measured fluorescence intensity, and F₀ is the initial fluorescence intensity in the absence of acrylamide. A, ○, 3 mM MgATP; ●, 3 mM MgATP and 10 μM daunorubicin; ▤, 3 mM MgATP and 10 μM 4’-epi-doxorubicin; □, 10 μM anthracycline derivative. B, ○ 3 mM Mg ATP; ●, 3 mM Mg ATPγS and 10 μM daunorubicin; ▤, 3 mM Mg ATPγS and 10 μM 4’-epi-doxorubicin; □, 10 μM anthracycline derivative. The results are the means of three experiments. The error bars represent the standard deviation.
anthracycline derivatives to determine whether ATP binding and hydrolysis still occurred. In the absence of anthracycline derivatives, an ATPase activity of 65 nmol/min/mg of protein was measured for the reconstituted P-glycoprotein. According to Table II, P-glycoprotein exhibited ATPase activity in the presence of all the derivatives tested, demonstrating that significant inhibition of ATP binding and hydrolysis did not occur when the drugs were bound to the protein.

Inhibition of $[^3H]$Azidopine Photolabeling of P-glycoprotein by Anthracycline Derivatives—Azidopine, an analog of verapamil, is able to specifically label P-glycoprotein (25). The ability to inhibit photoaffinity labeling has frequently been used as an indicator of whether a particular drug interacts with P-glycoprotein (26, 27). Therefore, to demonstrate the binding of the anthracycline derivatives to P-glycoprotein and to determine whether differences in azidopine competition could be identified between cytotoxic and noncytotoxic anthracycline derivatives, photolabeling experiments were performed on CHB30 plasma membrane vesicles in which about 15% of the protein is P-glycoprotein. Fig. 6 shows the densitometric analysis of the inhibition of $[^3H]$azidopine labeling in the presence of increasing concentrations (10, 30, and 100 $\mu$M) of each anthracycline derivative. Although all the compounds were able to compete with $[^3H]$azidopine labeling, the observed rate of inhibition varied as a function of the drug tested. In fact, at final concentrations of 30 and 100 $\mu$M, the three cytotoxic compounds (iododoxorubicin, 4-demethoxy-daunorubicin, and FCE) exhibited a stronger inhibition of $[^3H]$ azidopine labeling than noncytotoxic derivatives (daunorubicin and 4'-epi-doxorubicin). This suggests a higher affinity of these cytotoxic agents for P-glycoprotein or the binding of drugs to several P-glycoprotein sites.

**DISCUSSION**

Previous experiments, including infrared spectroscopy (18), enzymatic proteolysis (28, 29), fluorescence labeling in the NBD domains (17), and immunoreactivity experiments (30), have suggested that P-glycoprotein may exist in different conformational states during its catalytic cycle. Our data provide strong evidence that the protein undergoes tertiary conformational changes depending on the nature of the ligands.

Our experiments demonstrated that upon addition of MgATP, the enzyme adopts a different tertiary structure, resulting in a significantly increased solvent accessibility, according to previous data (18). MgATP-$\gamma$S, a nonhydrolyzable analog of MgATP, was used to investigate the structural changes associated with nucleotide binding. Our fluorescence measurements provide evidence that MgATP-$\gamma$S binding increases slightly but significantly, the accessibility of some P-glycoprotein domains. The structural change observed upon ATP bind-

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**TABLE I**

Influence of ligand binding on the Stern-Volmer constant ($K_{sv}$)

The Stern-Volmer constant was calculated using the Stern-Volmer equation. Concentrations of 10 $\mu$M for the anthracycline derivatives and of 3 mM in nucleotide were used in all cases.

| Ligand                                | $K_{sv}$ $\mu^{-1}$ |
|---------------------------------------|---------------------|
| None                                  | 0.555               |
| MgATP                                 | 1.415               |
| MgATP-$\gamma$S                       | 0.955               |
| Anthracycline derivatives             | 0                   |
| Noncytotoxic anthracyclines + nucleotide |                     |
| Daunorubicin + MgATP                   | 0.545               |
| 4'-Epi-doxorubicin + MgATP            | 0.6                 |
| Daunorubicin + MgATP-$\gamma$S        | 0.405               |
| 4'-Epi-doxorubicin + MgATP-$\gamma$S  | 0.645               |
| Cytotoxic anthracyclines + nucleotide |                     |
| Iododoxorubicin + MgATP               | 0.135               |
| 4-Demethoxy-daunorubicin + MgATP      | 0.01                |
| Iododoxorubicin + MgATP-$\gamma$S     | 0.185               |
| 4-Demethoxy-daunorubicin + MgATP-$\gamma$S | 0.27          |
| Cytotoxic FCE + nucleotide            |                     |
| FCE + MgATP                           | 0.625               |
| FCE + MgATP-$\gamma$S                 | 0.08                |

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**FIG. 5.** Stern-Volmer plots of P-glycoprotein Trp quenching by acrylamide upon co-addition of cytotoxic anthracycline derivatives and MgATP (A) or MgATP-$\gamma$S (B). $F$ is the measured fluorescence intensity, and $F_0$ is the initial fluorescence intensity in the absence of acrylamide. $\Box$, 3 mM MgATP and 10 $\mu$M 4-demethoxy-daunorubicin; $\Delta$, 3 mM MgATP and 10 $\mu$M FCE. $\Box$, 3 mM MgATP; $\bullet$, 3 mM MgATP-$\gamma$S and 10 $\mu$M iododoxorubicin; $\nabla$, 3 mM MgATP-$\gamma$S and 10 $\mu$M 4-demethoxy-daunorubicin; $\Delta$, 3 mM MgATP-$\gamma$S and 10 $\mu$M FCE. The results are the means of three experiments. The error bars represent the standard deviation.
ing (P-glycoprotein + MgATP) was much more pronounced when ATP hydrolysis occurred (P-glycoprotein + MgATP).

In this study, we also demonstrated the influence of the binding of five anthracycline derivatives to P-glycoprotein. Binding of the five anthracycline derivatives to P-glycoprotein reduces accessibility of the protein to solvent. The enzyme therefore undergoes a first conformational change. However, in the presence of MgATP or MgATP\(\gamma S\), addition of cytotoxic and noncytotoxic derivatives resulted in various degrees of Trp fluorescence quenching. In the presence of noncytotoxic derivatives (daunorubicin and 4'-epi-doxorubicin), binding of MgATP\(\gamma S\) was necessary and sufficient for the protein to undergo a conformational change. The protein was stabilized in a confirmation state intermediate between the "opened" structure observed in the presence of MgATP alone and the "closed" structure observed upon binding of the drug. In the presence of the cytotoxic ido-doxorubicin and 4-demethoxy-daunorubicin, neither ATP binding nor ATP hydrolysis were capable of modifying the tertiary structure of P-glycoprotein. The protein was maintained in the closed conformational state induced by the binding of drug derivative. However, the ATPase activity measurements described in this paper clearly indicated that ATP is still able to bind to P-glycoprotein and is hydrolyzed in the presence of the five anthracycline derivatives tested. In the presence of noncytotoxic agents, the protein probably undergoes a conformational change after ATP binding and hydrolysis, which might be an important step in the catalytic cycle of P-glycoprotein.

| Anthracycline derivative | ATPase activity \(\text{nmol/min/mg of P-glycoprotein}\) |
|--------------------------|-----------------------------------------------------|
| Daunorubicin             | 62 ± 1                                               |
| 4'-Epio-doxorubicin      | 44 ± 1                                               |
| Iododoxorubicin          | 98 ± 1                                               |
| 4-Demethoxy-daunorubicin | 66 ± 0.5                                             |
| FCE                      | 62 ± 0.5                                             |

\(^{2}\) A. B. Shapiro, unpublished data.

**FIG. 6.** Photoaffinity labeling of P-glycoprotein-containing plasma membrane vesicles with 0.3 \(\mu\)M azidopine in the presence of varying concentrations of anthracycline derivatives. The samples were run on an 8% Laemmli SDS-polyacrylamide gel. The gel was dried, amplified, and exposed to x-ray film (Kodak) for 1 day at \(-70^\circ\)C. Approximately half of the sample was lost.

\(^{2}\) A. B. Shapiro, unpublished data.

\(^{1}\) C. M. Privitera and H. R. Toole, unpublished data.
concentrations of each anthracycline derivative by fluorescence monitoring. According to these data, noncytotoxic daunorubicin and 4’-epi-doxorubicin inhibit rhodamine 123 transport but stimulate Hoechst 33342 transport. Cytotoxic idoxorubicin and FCE seem to bind to the Hoechst 33342 site as well as the rhodamine 123 site, whereas 4-demethoxy-daunorubicin binds more weakly to the Hoechst site. All together, these data suggest distinct binding sites for the cytotoxic and noncytotoxic drugs.

In conclusion, this study describes distinct P-glycoprotein conformations corresponding to different drug and ATP binding and hydrolysis states. Separate or simultaneous addition of MgATP and drug substrates give rise to distinct conformational changes in the P-glycoprotein molecule, confirming that coupling between the drug-binding site(s) and the catalytic sites for ATP hydrolysis occurs.

Interestingly, a difference in the protein comportment during these stages has been identified in the presence of cytotoxic and noncytotoxic derivatives. It is currently assumed that P-glycoprotein binds and transports substrates with a low specificity via a relatively nonspecific hydrophobic binding pocket (35). However, our results demonstrate that minor changes in the anthracycline structure cause major modifications in the specificity via a relatively nonspecific hydrophobic binding pocket.