Allosteric Modulation Bypasses the Requirement for ATP Hydrolysis in Regenerating Low Affinity Transition State Conformation of Human P-glycoprotein*

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ATP-dependent drug transport by human P-glycoprotein (Pgp, ABCB1) involves a coordinated communication between its drug-binding site (substrate site) and the nucleotide binding/hydrolysis domain (ATP sites). It has been demonstrated that the two ATP sites of Pgp play distinct roles within a single catalytic turnover; whereas ATP binding or/and hydrolysis by one drives substrate translocation and dissociation, the hydrolytic activity of the other resets the transporter for the subsequent cycle (Sauna, Z. E., and Ambudkar, S. V. (2001) J. Biol. Chem. 276, 11653–11661). Trapping of ADP (or 8-azido-ADP) and vanadate (ADP-Vi or 8-azido-ADP-Vi) at the catalytic site, following nucleotide hydrolysis, markedly reduces the affinity of Pgp for its transport substrate [125I]iodoarylazidoprazosin ([125I]IAAP), resulting in dissociation of the latter. Regeneration of the [125I]IAAP site requires an additional round of nucleotide hydrolysis. In this study, we demonstrate that certain thioxanthene-based allosteric modulators, such as cis-(Z)-flupentixol and its closely related analogs, induce regeneration of [125I]IAAP binding to vanadate-trapped (or fluoroaluminate-trapped) Pgp without any further nucleotide hydrolysis. Regeneration was facilitated by dissociation of the trapped nucleotide and vanadate. Once regenerated, the substrate site remains accessible to [125I]IAAP even after removal of the modulator from the medium, suggesting a modulator-induced relaxation of a constrained transition state conformation. Consistent with this, limited trypsin digestion of vanadate-trapped Pgp shows protection by cis-(Z)-flupentixol of two Pgp fragments (~60 kDa) recognizable by a polyclonal antiserum specific for the NH2-terminal half. No regeneration was observed in the Pgp mutant F983A that is impaired in modulation by flupentixols, indicating involvement of the allosteric modulator site in the phenomenon. In summary, the data demonstrate that in the nucleotide-trapped low affinity state of Pgp, the allosteric site remains accessible and responsive to modulation by flupentixol (and its closely related analogs), which can reset the high affinity state for [125I]IAAP binding without any further nucleotide hydrolysis.

The human P-glycoprotein (Pgp)* functions as an ATP-driven drug transporter conferring multidrug resistance in cancer cells and restricting bioavailability of many antimicrobial and anticancer agents (1, 2). It is a 1280-amino acid integral membrane protein of the ATP-binding cassette (ABC) transporter family (3, 4), with two highly homologous halves, each containing a hydrophobic transmembrane domain and a relatively hydrophilic cytosolic domain. Each hydrophobic domain contains six putative transmembrane helices that, in conjunction with the transmembrane regions of the other half, form the drug-translocating pathway (substrate site) across the lipid bilayer (1, 5). The cytosolic domains each contain three consensus sequences (3) that together contribute to the formation of two ATP binding/hydrolysis sites (ATP sites) (6).

Both ATP sites are essential for the drug transport function of Pgp (7–10) but are believed to hydrolyze ATP in an alternate sequence (11–13). Pgp possesses a basal rate of ATP hydrolysis that is stimulated upon interaction with many transport substrates as well as with several Pgp modulators (14). Binding and hydrolysis of ATP induce a conformational change in Pgp (15–17) that is coupled to substrate translocation across the lipid bilayer and its subsequent dissociation (18–24). The two ATP sites of Pgp were believed to be functionally equivalent with drug translocation and regeneration of the transporter directly coupled to a single round of ATP binding or/and hydrolysis (12, 25, 26). However, evidence suggests that although the two ATP sites assume similar structural conformation, they have distinct functional roles within a single catalytic turnover of Pgp (27–29). Experiments with transport substrate [125I]iodoarylazidoprazosin ([125I]IAAP) demonstrated that ATP binding and hydrolysis by one drives substrate translocation and dissociation (18, 19), whereas hydrolysis by the other resets the transporter for the subsequent round of transport activity (27, 28). Vanadate (Vi), a phosphate analog, replaces phosphate at the catalytic site (30) when present during ATP hydrolysis. Due to its low rate of dissociation, vanadate stabilizes Pgp in a catalytic intermediate (mimicking a conformational state) that immediately follows the first ATP hydrolytic event (11, 18, 19, 22, 23). Trapping of ADP and vanadate at the catalytic site, following ATP hydrolysis, leads to a dramatic decrease in the affinity of Pgp for its transport substrates, such as [125I]IAAP and [3H]azidopine, leading to substrate dissociation (18, 19). This is accompanied by an experimentally detectable conformational change (15, 17). Chemical cross-linking with thiol reagents and cysteine-scanning mutagenesis revealed increased accessibility of the drug-translocating pathway from the extracellular side of the lipid bilayer due to vanadate trapping (22).

Interestingly, the [125I]IAAP-binding site once transformed to its low affinity state remains unaltered even after dissociation of vanadate and

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* This work was supported by United States Public Health Services Grant GM067926 and Uniformed Services University of the Health Sciences Intramural Grant C071FU. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: Pgp, P-glycoprotein; ABC, ATP-binding cassette; IAAP, iodoarylazidoprazosin; Vi, vanadate; MES, 4-morpholineethanesulfonic acid.
ADP from the catalytic sites of Pgp (27, 28). Regeneration of the [\textsuperscript{125}I]IAAP site required an additional round of ATP hydrolysis by the catalytic domain, suggesting a tight coupling of functional activities between the ATP and the substrate sites. Binding of ATP does not provide the necessary drive for [\textsuperscript{125}I]IAAP site regeneration (27, 28).

We recently demonstrated that thioxanthen-based Pgp modulators interact at a site distinct from the site of [\textsuperscript{125}I]IAAP recognition and allosterically modulate Pgp function (31, 32). Of the thioxanthen-based modulators, the cis isomer of flupentixol (33) stimulates both [\textsuperscript{125}I]IAAP interaction and ATP hydrolysis by Pgp yet effectively inhibits substrate translocation. Inhibition occurs due to interference in [\textsuperscript{125}I]IAAP dissociation (32). Experimental evidence also suggests that the allosteric site can effectively communicate with the substrate and the ATP sites (31, 32). In this study, we demonstrate that the allosteric site remains accessible and responsive to modulator interaction, in the vanadate-trapped low affinity conformation of Pgp and mediates regeneration of [\textsuperscript{125}I]IAAP binding, bypassing the requirement for ATP hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Chemicals—cis-(Z)-Flupentixol** was from Research Biochemicals International. cis-(Z)-clopentixol and cis-(Z)-753 were generously provided by Dr. James M. Ford (Stanford University School of Medicine, Stanford, CA). [\textsuperscript{125}I]IAAP (2200 Ci/ml) was purchased from PerkinElmer Life Sciences. [\textsuperscript{125}I]IAAP (2200 Ci/ml) was purchased from Affinity Labeling Technologies, Inc. Nonradioactive 8-azido-ATP was purchased from ICN. All other chemicals were obtained from either Sigma or Bio-Rad. Pgp-specific polyclonal antibody PEPG13 was generously provided by Dr. Michael M. Gottesman (Laboratory of Cell Biology, National Cancer Institute).

**Baculovirus Expression of Human Pgp—Recombinant baculoviruses**BV-MDR1-(His)_6 (19) and BV-MDR1-(His)_6-F883A (32) harboring the wild type and the Pgp F883A mutant, respectively, were used to infect High Five insect cells grown in Excell 400 medium as described (34). Cells were grown to 80% confluence at 27 °C in monolayer, infected with the recombinant baculovirus with multiplicity of infection of 10, and harvested after 72 h of infection.

**Isolation of Crude Membranes From High Five Insect cells—**Crude membranes were prepared according to Dey et al. (18). Infected cells were harvested and washed twice in phosphate-buffered saline containing 1% aprotinin. Washed cells were incubated on ice for 45 min in homogenization buffer (50 mM Tris-HCl, pH 7.5, 50 mM mannitol, 2 mM EGTA, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1% aprotinin) and disrupted by repeated strokes of a Dounce homogenizer. Following homogenization, undisrupted cells and nuclei were removed by centrifugation at 500 × g for 20 min. The supernatant was collected and diluted with resuspension buffer (containing 50 mM Tris, pH 7.5, 300 mM mannitol, 1 mM EGTA, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1% aprotinin) and centrifuged at 100,000 × g for 1 h. The pellet was washed once with the same buffer and resuspended in resuspension buffer containing 10% glycerol by passing through a hypodermic needle (gauge size 19 and then 23). Membranes were stored at −70 °C in aliquots. Protein concentration was measured by a modified Lowry method (35) using bovine serum albumin as a standard.

**Recovery of [\textsuperscript{125}I]IAAP Binding to Vanadate-trapped and Fluoroualuminate-trapped Pgp—**Isolated insect cell membranes (0.1–0.15 mg/ml) were incubated at 37 °C for 5 min in ATPase assay buffer (50 mM MES, pH 7.0, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, and 10 mM MgCl\textsubscript{2}) containing 1.25 mM 8-azido-ATP and 0.25 mM sodium orthovanadate. The reaction was stopped by transferring the samples on ice, and the excess 8-azido-ATP as well as vanadate were removed by centrifugation at 200,000 × g for 15 min at 4 °C. The pellet was resuspended in 50 mM MES, pH 7.0, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, and 2 mM dithiothreitol divided into aliquots (15 μg of protein/100 μl); and incubated at 37 °C for varying periods of time. Wherever indicated, 25 μM Pgp modulators (cis-(Z)-flupentixol, cis-(Z)-clopentixol, cis-(Z)-753) or 1.25 mM 8-azido-ATP was added to the resuspended membranes 5 min prior to incubation at 37 °C. Incubation was stopped at the indicated time intervals; 5 mM [\textsuperscript{125}I]IAAP was added to each tube, incubated on ice for 5 min, and photocross-linked by UV irradiation for 5 min at room temperature. The photocross-linked samples were resolved by SDS-PAGE, and radioactivity associated with the Pgp band was quantified by PhosphoImager analysis and captured on an x-ray film for documentation.

Regeneration of fluoroualuminate-trapped Pgp was carried out similar to vanadate-trapped Pgp, except trapping was done in the presence of 0.5 mM AlCl\textsubscript{3}, plus 2.5 mM NaF.

**Dissociation of [\textsuperscript{125}I]-8-Azido-ATP from Pgp—**Isolated insect cell membranes (1 mg/ml) were incubated in ATPase assay buffer (50 mM MES, pH 7.0, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, and 10 mM MgCl\textsubscript{2}) containing 50 μM [\textsuperscript{125}I]-8-azido-ATP, and 0.25 mM sodium orthovanadatecaed at 37 °C under subdued light for 30 min. Reactions (vanadate trapping) were stopped by adding 12.5 mM ice-cold ATP and transferring the tubes immediately on ice. Following trapping, free nucleotides and sodium orthovanadate were removed by centrifugation at 200,000 × g for 15 min at 4 °C, and the pellet was resuspended in 50 mM MES, pH 7.0, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, and 2 mM dithiothreitol. The resuspended membranes were incubated at 37 °C, and at various times 100-μl aliquots (100 μg of protein) were transferred to ice and UV-photocross-linked at 365 nm for 10 min. Wherever indicated, 25 μM sodium orthovanadate and 25 μM cis-(Z)-flupentixol of Pgp modulator were added to the samples prior to incubation of the resuspended pellet at 37 °C. The photocross-linked samples were resolved by SDS-PAGE, and the radioactivity associated with the Pgp band was documented and quantified.

**Limited Trypsin Digestion of Vanadate-trapped Pgp—**Insect cell membranes (0.6 mg/ml) were vanadate-trapped at 37 °C for 5 min in the presence of 1.25 mM 8-azido-ATP and 0.25 mM sodium orthovanadate in ATPase assay buffer (final volume 2 ml). The reaction was stopped by adding equal volumes of 12 mM ice-cold ATPase buffer, and excess nucleotides and vanadate were removed by centrifugation at 300,000 × g for 15 min at 4 °C. Membrane pellet was resuspended in 1.86 ml of ice-cold resuspension buffer, containing 25 mM Tris-HCl, 300 mM mannitol, and 50 mM sodium chloride. Following resuspension, vanadate-trapped membranes were divided into 100-μl aliquots and incubated at 37 °C for varying periods of time with trypsin at 0.6 mg/ml final concentration. Wherever indicated, the vanadate-trapped membrane was incubated on ice for 5 min with 25 μM cis-(Z)-flupentixol prior to incubation with trypsin. Trypsin digestion was stopped by adding 5× SDS-PAGE sample buffer and incubation at room temperature for 30 min. Samples equivalent to 3 μg of membrane protein were resolved by SDS-PAGE, and peptide bands were detected by immunoblotting with Pgp-specific polyclonal antiserum PEPG13. The intensity of the major bands was quantified using densitometric software Image SXM.

**SDS-PAGE and Immunoblot Analysis—**Electrophoresis and immunoblot analysis were performed as described previously (36).

**RESULTS**

**cis-(Z)-Flupentixol Regenerates [\textsuperscript{125}I]IAAP Binding to Vanadate-trapped Pgp in the Absence of Nucleotide Hydrolysis—**The vanadate-trapped low affinity conformation of Pgp requires an additional round
of nucleotide hydrolysis to regain \(^{125}\text{I}\)IAAP binding to its substrate site. Since allosteric modulator cis-(Z)-flupentixol stimulates \(^{125}\text{I}\)IAAP interaction with Pgp through a site functionally distinct from the substrate site (31, 32), we investigated its ability to interact with the vanadate-trapped conformation and regenerate \(^{125}\text{I}\)IAAP binding to the substrate site.

\(^{125}\text{I}\)IAAP is a transport substrate for Pgp and can be conveniently photocross-linked by UV irradiation to its interaction site, allowing a snapshot of the functional property of the Pgp substrate site, both spatially and temporally. Although the photoaffinity labeling does not provide a stoichiometric binding, inhibition studies with other transport substrates (such as vinblastine) and competitive modulators (such as cyclosporin A) suggest that the affinity-labeled fraction is a fair representation of the entire Pgp population. Also, instead of ATP, we used 8-azido-ATP to be consistent with the experiments done in parallel to study (6-32P)8-azido-ADP trapping and dissociation.

As indicated in Fig. 1A, in the presence of 1.25 mM Mg8-azido-ATP, \(^{125}\text{I}\)IAAP binding to vanadate-trapped Pgp was completely regenerated, whereas no comparable regeneration was observed in the absence of any added nucleotide (Fig. 1, A and B). Interestingly, when vanadate-trapped membranes were incubated with 25 \(\mu\text{M}\) cis-(Z)-flupentixol in a vanadate- and nucleotide-free medium, a complete regeneration of \(^{125}\text{I}\)IAAP binding was observed within 2.5 min of incubation at 37°C (Fig. 1, A and B). To avoid the possibility of any residual 8-azido-ATP being hydrolyzed during incubation with cis-(Z)-flupentixol, no Mg\(^{2+}\) was added, and the reaction was supplemented with 0.25 mM EDTA. No modulator-induced regeneration of the \(^{125}\text{I}\)IAAP binding was observed in the Pgp F983A mutant that is impaired in interaction with cis-(Z)-flupentixol (Fig. 1, A (bottom) and B). Therefore, the results suggest that even in the vanadate-trapped conformation of Pgp, the allosteric site is accessible to modulator interaction and capable of regenerating \(^{125}\text{I}\)IAAP binding to the substrate site in the absence of nucleotide hydrolysis.

It was not totally clear why the regenerated level of \(^{125}\text{I}\)IAAP binding was higher than that bound to untrapped Pgp. One possible explanation is that since both the untrapped and trapped Pgp were subjected to similar washing and centrifugation steps, some of the untrapped Pgp molecules get inactivated during the procedure, whereas the tightly bound ADP and vanadate at the catalytic site conferred protection to the trapped Pgp from such inactivation. Ligand-induced protection of enzymatic activity is a well known phenomenon.
Similar to vanadate, fluoroaluminate in combination with ATP inhibits Pgp ATPase activity, which involves trapping of nucleotide in catalytic sites (37). Fluoroaluminate-trapped Pgp also showed reduced [\(^{125}\)I]IAAP binding, indicating formation of a low affinity transition state (Fig. 2). Consistent with our observation in vanadate-trapping experiments, regeneration of [\(^{125}\)I]IAAP binding was achieved both in the presence of 1.25 mM 8-azido-ATP (up to 90%) and 25 \(\mu\)M cis-(Z)-flupentixol (up to 100%). Therefore, the effect of cis-(Z)-flupentixol was not limited to vanadate-trapped Pgp and was reproducible using a different mode of trapping. We also observed that structurally related thioxanthene derivatives, cis-(Z)-clopentixol and cis-(Z)-753, were able to induce 75–100% regeneration of [\(^{125}\)I]IAAP binding to fluoroaluminate-trapped Pgp, respectively (Fig. 2).

Modulator-induced Regeneration of [\(^{125}\)I]IAAP Binding Is Facilitated by Dissociation of the Trapped ADP and Vanadate—To understand the mechanism by which allosteric modulator cis-(Z)-flupentixol regulates [\(^{125}\)I]IAAP binding to vanadate-trapped Pgp, it is important to determine whether dissociation of 8-azido-ADP and vanadate has to precede regeneration. Although vanadate and [\(\alpha\)-32P]8-azido-ADP have been shown to dissociate spontaneously from the vanadate-trapped Pgp upon incubation at 37°C (Fig. 3A), as expected, vanadate alone was unable to regenerate [\(^{125}\)I]IAAP binding (Fig. 3B). This suggests that substrate site regeneration by cis-(Z)-flupentixol is facilitated by dissociation of trapped 8-azido-ADP and vanadate from the catalytic site.

One possibility is that in the vanadate-trapped conformation, the second ATP site remained occupied by unhydrolyzed 8-azido-ATP with magnesium, which was occluded from EDTA and stimulated by the occluded nucleotide. Because the ATPase activity was inhibited by the occluded nucleotide, it was important to determine whether dissociation of 8-azido-ADP and vanadate has to precede regeneration. Although vanadate and [\(\alpha\)-32P]8-azido-ADP have been shown to dissociate spontaneously from the vanadate-trapped Pgp upon incubation at 37°C (Fig. 3A), as expected, vanadate alone was unable to regenerate [\(^{125}\)I]IAAP binding (Fig. 3B). This suggests that substrate site regeneration by cis-(Z)-flupentixol is facilitated by dissociation of trapped 8-azido-ADP and vanadate from the catalytic site.

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The results indicate that incubation with modulator-free medium and assessed for substrate site regeneration. For 5 min, the vanadate-trapped Pgp was removed by centrifugation to a modulator-free medium. Vanadate-induced 8-azido-ADP-trapped Pgp, following dissociation, membranes were incubated at 37 °C for another 30 min in the presence of either 0.25 mM sodium orthovanadate (VII), 0.25 mM sodium orthovanadate plus 25 μM cis-(Z)-flupentixol (V + Cis(Z)), 0.25 mM sodium orthovanadate plus 0.25 mM EDTA (V + EDTA), or 0.25 mM sodium orthovanadate plus 25 μM cis-(Z)-flupentixol plus 0.25 mM EDTA (V + Cis(Z) + EDTA). After incubation, samples were photocross-linked for 30 min on ice, 33 μg of protein/sample were resolved by SDS-PAGE, and radioactivity associated with Pgp was captured in an x-ray film (autoradiogram) (A). For each lane, Pgp-associated radioactivity was quantified in a PhosphorImager and expressed as the percentage of [α-32P]8-azido-ADP bound to vanadate-trapped Pgp prior to dissociation. The values represent the average of two independent experiments.

Susceptibility to limited trypsin digestion has been a useful technique for determining conformational changes in Pgp induced by nucleotides and transport substrates (15, 16, 38). The most susceptible trypsin site of Pgp is in the linker region connecting the two halves of the protein (Site 1 in Fig. 8C) at Arg680 (39). In crude membrane preparations, this site is endogenously cleaved by a contaminating protease when incubated at 37 °C, yielding an 80-kDa NH2-terminal fragment and a 60-kDa COOH-terminal fragment. Although only a small fraction of the total Pgp molecules are cleaved endogenously, the 80-kDa fragment appears as a significant band in an immunoblot when probed with Pgp-specific antibody and exposed for a fairly long period of time (Fig. 8A, first two lanes).

To detect any possible conformational change involved in cis-(Z)-flupentixol-induced regeneration of the high affinity state of Pgp from vanadate-trapped conformation, we studied the effect of cis-(Z)-flupentixol on susceptibility to limited trypsin digestion of vanadate-trapped Pgp. Results indicated that 25 μM cis-(Z)-flupentixol conferred a clear protection to two Pgp fragments with molecular mass of ~60 kDa detectable by a polyclonal antiserum PEPG13 specific to the NH2-terminal half of Pgp (Fig. 8, A and B). PEPG13 was raised against a recombinant peptide with amino acid residues 592–636 of human Pgp present as a COOH-terminal fusion with Pseudomonas endotoxin (40). No protection of the 140-kDa full-length Pgp or of the 80-kDa NH2-terminal fragment was observed. Therefore, the results suggested that a distinct conformational state was induced or stabilized by interaction of vanadate-trapped Pgp with cis-(Z)-flupentixol.

A similar experiment with untrapped Pgp demonstrated protection of the 80-kDa fragment, in addition to the 60-kDa doublet, from trypsin digestion by 25 μM cis-(Z)-flupentixol (Fig. 8A, last two lanes). The appearance of the doublet suggests two closely spaced trypsin sites in...
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**FIGURE 6.** Regeneration of $^{125}$I-IAAP binding by cis-(Z)-flupentixol is irreversible.

Pgp-containing membranes (0.15 mg/ml protein) were vanadate-trapped with 1.25 mm B-azido-ATP and 0.25 mm sodium orthovanadate. Vanadate-trapped membranes were pelleted and resuspended in a vanadate- and nucleotide-free medium and incubated at room temperature for 5 min with 25 μM cis-(Z)-flupentixol. Following incubation, cis-(Z)-flupentixol was removed by pelleting and resuspending the membranes in a modulator-free assay buffer, prior to incubation at 37 °C with 5 nM $^{125}$I-IAAP for the indicated time periods, and photocross-linked. Photocross-linked samples (3 μg of protein/well) were then resolved by SDS-PAGE, and radioactivity associated with Pgp was captured in an x-ray film (A) and quantified using a PhosphorImager (B). Radioactivity associated with Pgp was expressed as the percentage of $^{125}$I-IAAP bound to untrapped Pgp. The values are averages of two independent experiments.

The modulator had no effect on the enzymatic activity of trypsin as determined using chromogenic substrate $N$-a-benzoylarginine-$L$-P-nitroanilide.

**DISCUSSION**

It was recently demonstrated that trapping of ADP-Vi in the catalytic site of Pgp following ATP hydrolysis dramatically reduces the affinity of Pgp for its transport substrate $^{[125]}$I-IAAP (18, 19). The low affinity state of Pgp is described as a catalytic intermediate state that follows immediately after ATP hydrolysis with the substrate molecule translocated and dissociated. However, regeneration of $^{[125]}$I-IAAP binding to vanadate-trapped Pgp cannot be induced even after complete dissociation of the trapped nucleotide and vanadate; instead, it required an additional round of nucleotide hydrolysis (Fig. 1, A and B) (27). These results suggested that the $^{[125]}$I-IAAP site remains occluded in the vanadate-trapped conformation and only becomes accessible after a second hydrolytic event (28). We reported that thioxanthene-based Pgp modulators, such as flupentixols, allosterically interact with Pgp through a site that is functionally distinct from the site of substrate ($^{[125]}$I-IAAP) recognition (32). In this study, we demonstrate that in the vanadate-trapped low affinity conformation of Pgp, the allosteric site remains accessible and responsive to allosteric modulation by cis-(Z)-flupentixol (Fig. 1, A and B). The modulation involves a change in Pgp conformation (Fig. 8, A and B), leading to regeneration of the $^{[125]}$I-IAAP site without any requirement for additional nucleotide hydrolysis.

The thioxanthene derivative cis-(Z)-flupentixol and its closely related analogs (such as cis-(Z)-clopentixol and cis-(Z)-753) inhibit Pgp-mediated drug transport in an allosteric manner, in which the residue Phe983 plays a crucial role (32). Although the binding site of the allosteric modulator is yet to be defined, the functional distinctness of the site is becoming increasingly apparent. The stimulatory effect of cis-(Z)-flupentixol ($K_D \sim 5 \mu M$) on $^{[125]}$I-IAAP binding to Pgp yet remained accessible and responsive to modulation by cis-(Z)-flupentixol, it clearly establishes a functional and spatial distinctness of the modulator interaction site. Recent work by Loo and Clarke (22) has demonstrated that the major substrate-binding site of Pgp is solvent-accessible in the vanadate-trapped conformation, presumably on the extracellular side, and suggested a role of incoming water molecules in the breaking of hydrogen bonds between the amino acid residues in the drug translocating pathway and the transport substrate molecules. This disruption of hydrogen bonding has been proposed as a general mechanism facilitating drug dissociation from Pgp (22). The ability of cis-(Z)-flupentixol to modulate vanadate-trapped Pgp suggests that either its interaction site does not fall within the con-
ventional substrate site or the interaction between cis-(Z)-flupentixol and Pgp is not perturbed by incoming water molecules.

The data indicate that once induced, the [125I]IAAP site remained regenerated even when the allosteric modulator cis-(Z)-flupentixol was removed from the medium (Fig. 6). One possibility is that Pgp-bound cis-(Z)-flupentixol does not dissociate readily, and as a result the regenerating effect on the [125I]IAAP site is sustained even in the absence of modulator in the medium. However, the stimulatory effect of cis-(Z)-

FIGURE 8. Regeneration by cis-(Z)-flupentixol alters trypsin susceptibility of vanadate-trapped Pgp. A, 0.6 mg/ml isolated insect cell membranes expressing wild type Pgp were vanadate-trapped with 5 mM ATP and 0.25 mM sodium orthovanadate in ATPase assay buffer. Following trapping, membranes were pelleted and resuspended in vanadate- and nucleotide-free medium. Resuspended membranes (60 μg/100-μl aliquot) were incubated with trypsin (80 μg/μl aliquot) at 37 °C either in the presence or in the absence of 25 μM cis-(Z)-flupentixol for varying times. Reactions were stopped by the addition of SDS-PAGE sample buffer and incubation at room temperature for 30 min. Samples were resolved by SDS-PAGE, and the proteolytic fragments of Pgp were detected in an immunoblot using Pgp-specific polyclonal antibody PEPG13. 3 μg of protein was loaded in each well. Similar trypsin treatment was carried out for 15 min at 37 °C on untrapped Pgp in the presence or in the absence of 25 μM cis-(Z)-flupentixol and resolved likewise (Untrapped + Trypsin, last two lanes), B, the density of the bands was determined using the software Image SXM (version 174). Band intensity was expressed in arbitrary units and plotted against time of incubation with trypsin. The values are the average of two independent experiments. C, a schematic representation of the possible tryptic fragments detected by PEG13. The arrows indicate the positions of the trypsin cleavage sites relevant to the study. The double-headed arrow shows the region corresponding to the PEPG13 epitope. The 140-kDa fragment represents the full-length Pgp, whereas the 80-kDa fragment is the product of the first trypsin cleavage at site 1, and the 60-kDa doublet is the product of subsequent cleavages at sites 2 and 3. The open ellipses represent the approximate nucleotide binding sites. The figure is drawn on an arbitrary scale.
Allosteric Modulation of Pgp (ABCB1)

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[125]IAAP binding to untrapped Pgp is abolished upon removal of the modulator from the medium, indicating no residual cis-(Z)-flupentixol after the centrifugation step and that the interaction between cis-(Z)-flupentixol and Pgp is reversible (Fig. 7). A more reasonable explanation would be that the vanadate-trapped state of Pgp represents an energetically constrained conformation, which upon interaction with cis-(Z)-flupentixol is transformed to a more energetically favorable state with the [125]IAAP site regenerated. The energy constraints of the vanadate-trapped conformation of Pgp have been proposed and experimentally demonstrated by other groups (12, 25, 26).

A change in conformation during cis-(Z)-flupentixol-induced regeneration of the [125]IAAP site is apparent from the altered susceptibility of vanadate-trapped Pgp to proteolytic cleavage in the presence of cis-(Z)-flupentixol (Fig. 8). Limited trypsin digestion of the vanadate-trapped Pgp demonstrates a protection by cis-(Z)-flupentixol of two Pgp fragments with molecular mass of ~60 kDa (Fig. 8A). The nature of this conformational change is distinctly different from that induced by cis-(Z)-flupentixol to untrapped Pgp, where the major protected Pgp fragment had an approximate molecular weight of 80 kDa (Fig. 8). This differential protection by cis-(Z)-flupentixol suggests that its modulatory effect on trapped and untrapped Pgp are not identical. This is consistent with the observation that cis-(Z)-flupentixol stimulates [125]IAAP binding to untrapped Pgp, whereas it simply regenerates the basal level of binding to the trapped conformation. However, both modulatory effects were mediated through interaction of cis-(Z)-flupentixol at the same site, since the residue Phe983 seems to play a crucial role in both phenomena (Fig. 1A, bottom) (31).

In ABC transporters, nucleotide-induced transition between the high and the low affinity (for substrate) conformations is believed to be the basic mechanism of energy-dependent vectorial transport. In Pgp, considerable controversy remains as to whether the transition from high to low affinity state (driving translocation) is coupled to ATP binding, to ATP hydrolysis, or to ADP dissociation (18–24, 27, 28). Irrespective of that, there is a general consensus in favor of an absolute requirement for ATP hydrolysis in resetting the transporter from its low affinity to high affinity conformation, which suggests a tight functional coupling between the two events. The fact that cis-(Z)-flupentixol effectively regenerates [125]IAAP binding to the vanadate-trapped low affinity conformation of Pgp, bypassing the requirement for ATP hydrolysis (Fig. 1), suggests an uncoupling effect associated with the modulator. Since fluoromethane traps both dinucleotide and trinucleotide at the catalytic site (37), the ability of cis-(Z)-flupentixol to regenerate [125]IAAP binding to fluoromethane-trapped Pgp indicates that irrespective of whether the low affinity state is generated by nucleotide hydrolysis (18, 19) or by tight nucleotide binding (21), transition to the high affinity state can be induced by the modulator. An argument can be made that the recovery of [125]IAAP binding by cis-(Z)-flupentixol to vanadate-trapped Pgp is not a true regeneration of the transport substrate site but instead reflects cis-(Z)-flupentixol-stimulated binding of [125]IAAP to a small population of untrapped Pgp in the reaction. The fact that regeneration of the transport substrate site by cis-(Z)-flupentixol required removal of 8-azido-ATP and vanadate rules out this possibility.

The spatial relationship of the fluoromethane interaction site with respect to the substrate-binding region of Pgp remains to be fully understood. The stimulatory effect of cis-(Z)-flupentixol on [125]IAAP binding suggested no physical overlap between the interaction sites of the two (18, 32). In this report, we demonstrate that in a catalytic transition state conformation of Pgp, which prevents binding of transport substrate [125]IAAP, the interaction site for flupentixol remains accessible and responsive to modulation. Although it is too premature to extend our conclusions drawn from the studies on [125]IAAP binding to the substrate site in general, the data are indicative of a nonconventional mode of action for cis-(Z)-flupentixol in modulating Pgp function.

Furthermore, according to a two-dimensional helical wheel projection map of the Pgp transmembrane regions by Loo and Clarke (5, 41), Phe983, which has a crucial role in modulation by flupentixol (Fig. 1) (18, 32), lies outside the major drug-binding pocket of Pgp. This encourages us to propose a spatially distinct site of interaction for these thioxanthenone derivatives, which can modulate Pgp function allosterically. Modulatory responses vary depending on the functional state of the transporter, which include uncoupling of the [125]IAAP-site regeneration from the catalytic activities in ATP-binding sites. It is interesting to observe that under both circumstances, cis-(Z)-flupentixol facilitated [125]IAAP binding to Pgp yet inhibits transport (32). We propose that in the presence of the modulator, substrate dissociation becomes the rate-limiting step, inhibition of which by cis-(Z)-flupentixol prevents transport.

The fact that a nonnucleotide ligand, such as cis-(Z)-flupentixol, can substitute for nucleotide hydrolysis in resetting the high affinity state of Pgp from its low affinity transition may provide important insight into the mechanism by which the transition between the low and the high affinity states takes place in Pgp as well as in other ABC transporters. Studies along this line are currently being pursued in our laboratory.

Acknowledgments—We thank Dr. Michael Gottesman and Dr. James M. Ford for encouragement and support and Dr. Carl Mann and Dr. Ernest Maynard for critical assessment of the manuscript.

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