Identification of Two Different States of P-glycoprotein in Its Catalytic Cycle

ROLE OF THE LINKER REGION IN THE TRANSITION BETWEEN THESE TWO STATES

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P-glycoprotein (Pgp) is a drug-translocating ATPase responsible for multidrug resistance in cancer. Although it is well-established that Pgp exhibits drug-dependent ATPase and ATP-dependent drug transport functions, the mechanism by which these two reactions are coupled remains unclear. We have shown recently that proteolytic cleavage of the linker region, which joins the NH₂ and COOH halves of the Pgp molecule, results in a Pgp form that exhibits drug-independent and ATP-dependent ATPase activities (Nuti et al., (2000) Biochemistry 39, 3424-3432; Nuti, S. L., and Rao, U. S. (2002) J. Biol. Chem. 277, 29417-29423). To understand the mechanism underlying this phenomenon, we used the procedure of vanadate-mediated trapping of the Pgp transport cycle intermediates to determine the steps in the catalytic cycle that are being regulated by the linker region. We show that vanadate stably traps Pgp under two different conditions, one in the presence of ATP alone and the other in the presence of ATP and drug, suggesting the existence of two Pgp conformations. These two conformations, one mediating basal and the other drug-stimulated ATPase reactions, represent different transport cycle intermediates of Pgp, because arresting Pgp in either conformation prevents the catalytic cycle from proceeding to completion. The results also show that these two conformations are uncoupled and appear simultaneously in Pgp that was cleaved in the linker region. These results together suggest that Pgp assumes at least two distinct conformational states, which catalyze two ATP hydrolysis events in the drug transport cycle, and the linker region mediates the transition between these two states of Pgp.

The phenomenon of multidrug resistance in cancer cells results from the overexpression of P-glycoprotein (Pgp) in their plasma membrane (1–3). The human Pgp is a 1280 amino acid polypeptide, and computer-assisted predictive algorithms indicate that it folds into two homologous halves, the NH₂-half and the COOH-half, which are joined by a cytoplasmically located stretch of ~75 amino acids (residues 633–709), commonly known as the linker region (4–6). Both of these halves contain six putative transmembrane segments and an ATP-binding site (3, 5). Demonstrations that Pgp binds anticancer drugs (7–9), mediates ATP-dependent drug uptake (10, 11), and exhibits ATPase activity in the presence of drugs that are known to be extruded by Pgp (12–15), and certain mutations in Pgp alter both the ATPase and drug transport functions (16–20), have led to the conclusion that the drug transport and ATP hydrolysis functions of Pgp are coupled. However, the mechanism of coupling is unclear.

To understand the mechanism of coupling, it is first necessary to identify various intermediate structures of Pgp that are presumably formed during the catalytic cycle. Toward this end, we have initiated studies to detect conformational differences in Pgp that was bound to ATP and transport substrate by using limited proteolysis combined with immunoblotting analyses. Although these studies did not provide any striking differences in the pattern of proteolysis of Pgp that was bound to different ligands, it was noticed that the proteolyzed Pgp exhibits ATPase activity both in the presence of drugs (drug-stimulated ATPase function) and absence of drugs (basal ATPase function). We have characterized this phenomenon using trypsin, chymotrypsin, and proteinase K (21, 22). The NH₂-terminal amino acid sequencing of the tryptic and chymotryptic peptides originating from the COOH-half of Pgp has identified, respectively, residues 680 and 682 as the sites cleaved by these proteases (22). These identified residues are interestingly located in the linker region, which led to the suggestion that the linker region is the only region in the entire molecule that is predominantly accessible for protease action, and cleavage of this region leads to the formation of a Pgp form that exhibits the basal ATPase activity.

To investigate the mechanism by which linker-region cleavage leads to the appearance of basal ATPase function, we used the procedures of sodium orthovanadate (Vi)-mediated trapping of nucleotide in the active site of Pgp, originally developed by Senior for investigating the Pgp catalytic cycle (23, 24). The results presented in this report show that Pgp exists at least in two different conformational states, which differ in the accessibility of the drug-binding site. These Pgp forms are intermediates of the catalytic cycle, one conformation that binds drug appears to mediate the drug-stimulated ATPase function, and the second conformation, which does not bind drug, appears to mediate the basal ATPase function. The results presented here also indicate that these two conformational states are detectable in the linker-region-cleaved Pgp, suggesting that the linker region is a key player in coupling these two states.
EXPERIMENTAL PROCEDURES

Preparation of Pgp-containing Membranes—The Sf9 insect cells were infected with a recombinant baculovirus carrying the human wild-type Pgp cDNA, and the total membrane fraction was prepared as described previously (17). Membranes prepared from Sf9 insect cells infected with a recombinant baculovirus carrying the α-subunit cDNA of the amiloride-sensitive sodium channel (25) were used as the control.

Pgp ATPase Activity Measurements—The basal and the verapamil-stimulated Pgp ATPase activities were measured by determining colorimetrically the inorganic phosphate released from ATP as described previously (21). The Pgp membrane preparations used in this study contained ~66 nmol/min/mg of verapamil-stimulated ATPase activity, and the linker region-cleaved Pgp exhibited the basal and verapamil-stimulated ATPase activities of ~22 and ~40 nmol/min/mg, respectively. These activities were considered 100%, and all the ATPase activities reported here are expressed as a percentage of these values.

Vi-induced Trapping of the Pgp ATPase—The Vi-induced trapping of the Pgp ATPase reactions were carried out as described previously (26) with the following modifications. Pgp membranes (~200 μg of membrane protein) were incubated either at 22°C or at 37°C for 5 min in 0.4 ml of 50 mM Tris-Cl buffer, pH 7.4, containing 0.1 mM Vi, 50 μM verapamil, 10 mM Mg-salt of ATP, AMP-PCP or ADP, and 10 mM Pi (Na2HPO4), as indicated in each experiment. The preincubation mixtures were centrifuged in a microcentrifuge at 14,000 rpm for 10 min at 4°C, and the membrane pellets were washed once in ice-cold 50 mM Tris-Cl buffer, pH 7.4. The pelleted membrane fraction was resuspended in Tris-glycerol buffer (50 mM Tris, pH 7.4, 50 mM mannitol, 2 mM EGTA, and 30% glycerol) and used in the ATPase activity measurements as described (21).

[125I]IAAP Photoaffinity Labeling—Photoaffinity labeling of membranes with [125I]IAAP was carried out by following the general procedure of Dey et al. (27). Briefly, the membranes (30–50 μg) containing full-length or trypsin-cleaved Pgp (membrane protein/trypsin at a ratio of 1000:1 w/w for 30 min at room temperature) were incubated with 8.3 μM [125I]IAAP, 0.1 mM Vi, and 10 mM MgATP, MgADP and P, or the non-hydrolyzable ATP analog, AMP-PCP, in 50 mM Tris-HCl buffer, pH 7.4, for 5 min at either 22°C or 37°C. The samples were then illuminated with 365 nm of ultraviolet light using a hand-held ultraviolet lamp (UVP Products, Upland, CA) for 5 min. The proteins in these reaction mixtures were precipitated with 8% (w/v) trichloroacetic acid and separated on 7.5% SDS-PAGE. The gels were dried and exposed to phosphor-screen at ~80°C for 14 h. Radioactivity incorporated into Pgp was detected by the STORM 840 PhosphorImager, and the intensity of the bands in the fluorograms was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Vi-induced Stable Inhibition of Pgp ATPase at 22°C Requires ATP and a Drug—Using the principle first developed by Senior, which was subsequently adapted by others (23, 24, 26, 28–32), that Vi induces stable inhibition of Pgp ATPase activity in the presence of MgADP, we have initiated experiments to characterize the effects of Vi on the Pgp ATPase activity in the presence of various ligands at 22°C. Pgp membranes were incubated with Vi and other ligands at 22°C for 5 min, and the membranes were collected by centrifugation as described under “Experimental Procedures.” The verapamil-stimulated ATPase activity in the membranes thus obtained was measured, and the results are shown in Fig. 1A. When Pgp was incubated with Vi alone, or Vi and MgATP, no loss of the verapamil-stimulated Pgp ATPase activity was observed. In the presence of Vi, MgATP and verapamil, nearly 70% of the verapamil-stimulated Pgp ATPase activity was inhibited. Interestingly, when MgATP in this preincubation reaction was replaced with MgADP and Pi, or MgAMP-PCP, no significant loss of the verapamil-stimulated Pgp ATPase activity was noticed. To further establish that the inhibition is dependent on the presence of verapamil, the above analysis was carried out by including a range of verapamil concentrations in the preincubation mixture containing fixed amounts of Pgp, Vi, and MgATP, and the verapamil-stimulated Pgp ATPase activity was measured. The results show (Fig. 1B) that the Pgp ATPase inhibition is verapamil concentration-dependent. Similar results were obtained upon replacing verapamil in the preincubation mixtures with vinblastine or cyclosporin A (not shown). Because all of these incubations were carried out at identical temperature, i.e. 22°C, and Vi-induced inhibition of Pgp ATPase activity selectively occurs in the presence of MgATP and drug, these results suggest that it is the participating ligands, i.e. the drug and the ATP, that determine the inhibition of Pgp.

Stable Inhibition of Pgp ATPase at 37°C Occurs in the Presence of ATP and Does Not Require Drug—In contrast to the above and to the previous reports (26, 28, 33), it is also known that Vi induces stable inhibition of Pgp ATPase in the presence of MgATP, and such inhibition does not require drug (23, 31, 34). Although the experimental details in these reports appeared comparable, one striking difference is the preincubation
A mixture was replaced with 10 mM MgADP and 10 mM Pi, or 10 mM MgATP. Where indicated in the figure, A was unaffected when Pgp was incubated with Vi (Fig. 2A). The verapamil-stimulated ATPase activity shown in Fig. 2A was measured in these membranes; the results are incorporated at 37 °C, which was either 22 °C or 37 °C.

Membranes were preincubated with Vi and other ligands as described (21). Pgp cleaved in the linker region was included, or MgATP in the preincubation mixture containing fixed amounts of Pgp and Vi, and the verapamil-stimulated Pgp ATPase activity in the resulting membranes was measured. Fig. 2B shows that the inhibition of Pgp ATPase activity is linear and dependent on the concentration of MgATP. These results suggested that Vi induces stable inhibition of Pgp ATPase at 37 °C in the presence of MgATP, and such inhibition does not require drug. Because all of the incubations of Fig. 2A were carried out at identical temperature, i.e. 37 °C, and the Vi-induced inhibition of Pgp ATPase activity occurs with a minimal requirement of MgATP, these results suggest, as pointed above, that it is the participating ligand, the hydrolysable ATP, that determines the inhibition of Pgp ATPase.

**Conditions for Stable Inhibition of Basal and Drug-stimulated ATPase Activity of the Linker Region-cleaved Pgp by Trypsin—** As reported previously, Pgp cleaved in the linker region by either trypsin, chymotrypsin, or proteinase K exhibits both the basal and the drug-stimulated ATPase activities (21, 22).

To understand the mechanism by which these two activities are elicited, Pgp-containing membranes were treated with trypsin at a ratio of 1000:1 (w/w) for 1 h, under which conditions Pgp was cleaved in the linker region and exhibited both basal and drug-stimulated ATPase activities, as reported previously (21). Pgp in which its linker region was cleaved by trypsin was preincubated at 22 °C with Vi in the presence of various ligands, and the basal and the verapamil-stimulated ATPase activities were measured. Fig. 3A shows that nearly 90% of the basal ATPase activity of the linker region-cleaved Pgp was inhibited upon preincubation at 22 °C with Vi and MgATP (Fig. 3A, hatched column). Under these experimental conditions, nearly 50% of the verapamil-stimulated ATPase activity remained unaffected (Fig. 3B, hatched column). To further determine whether the verapamil-stimulated ATPase activity can be completely inhibited in the presence of MgATP and Vi, the linker region-cleaved Pgp was incubated with increasing concentrations of MgATP in the presence of Vi. Measurements of ATPase activities in the resulting membranes, shown in Fig. 3C, indicate that the verapamil-stimulated ATPase activity can only be inhibited partially, amounting to ~60% under these experimental conditions (Fig. 3C, open circles). However, under the same conditions, complete inhibition of the basal ATPase activity is observed (Fig. 3C, filled circles). We next determined whether inclusion of drug in the preincubation mixture containing MgATP and Vi can result in a complete inhibition of the verapamil-stimulated ATPase activity of the linker region-cleaved Pgp. The results show that although complete inhibition of the basal ATPase activity is maintained (Fig. 3A, solid column), only ~60% of the verapamil-stimulated ATPase activity is inhibited (Fig. 3B, solid column). Because the Vi-induced inhibition of Pgp ATPase activity is known to be reversible (23, 26), the absence of complete inhibition of the verapamil-stimulated ATPase activity in the preincubation mixture containing Vi, MgATP, and drug is probably attributable to decreased stability of the complex as a result of trypsin-mediated cleavage of the linker region.

**Conditions for Stable Inhibition of Basal and Drug-stimulated ATPase Activity of the Linker Region-cleaved Pgp by Chymotrypsin—** We have shown recently that chymotrypsin also cleaves Pgp predominantly in the linker region, and such cleavage leads to the formation of a Pgp form that exhibits both the basal and drug-stimulated ATPase activities, similar to the effects of trypsin on Pgp (22). Interestingly, one of sites that is cleaved by chymotrypsin is residue 682, which is only 2 amino acids farther from a site that was cleaved by trypsin (22). Thus, to further establish the observations presented in Fig. 3, Pgp in

**Fig. 2. Stable inhibition of Pgp ATPase at 37 °C occurs in the presence of ATP and does not require drug.** A. Pgp membranes were incubated at 37 °C for 5 min in 50 mM Tris-Cl buffer, pH 7.4, containing 0.1 mM Vi and 10 mM MgADP, and error bars represent the S.D. The data represent the mean value of four experiments, and error bars represent the S.D. temperature, which was either 22 °C or 37 °C. To determine whether temperature influences the Vi-induced inhibition, Pgp membranes were preincubated with Vi and other ligands as described above except that the preincubation mixtures were incubated at 37 °C for 5 min. The membranes were collected by centrifugation, and the verapamil-stimulated Pgp ATPase activity in these membranes was measured; the results are shown in Fig. 2A. The verapamil-stimulated ATPase activity was unaffected when Pgp was incubated with Vi (Fig. 2A, open column) or with MgATP in the absence of added ligand was considered as 100%. The data represent the mean value of four experiments, and error bars represent the S.D.

To further establish that Pgp ATPase inhibition occurs in the presence of MgATP without the need for a drug, a range of MgATP concentration was included in the preincubation mixture containing fixed amounts of Pgp and Vi, and the verapamil-stimulated Pgp ATPase activity in the resulting membranes was measured. Fig. 2B shows that the inhibition of Pgp ATPase activity is linear and dependent on the concentration of MgATP. These results suggested that Vi induces stable inhibition of Pgp ATPase at 37 °C in the presence of MgATP, and such inhibition does not require drug. Because all of the incubations of Fig. 2A were carried out at identical temperature, i.e. 37 °C, and the Vi-induced inhibition of Pgp ATPase activity occurs with a minimal requirement of MgATP, these results suggest, as pointed above, that it is the participating ligand, the hydrolysable ATP, that determines the inhibition of Pgp ATPase.

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**Conditions for Stable Inhibition of Basal and Drug-stimulated ATPase Activity of the Linker Region-cleaved Pgp by Chymotrypsin—** We have shown recently that chymotrypsin also cleaves Pgp predominantly in the linker region, and such cleavage leads to the formation of a Pgp form that exhibits both the basal and drug-stimulated ATPase activities, similar to the effects of trypsin on Pgp (22). Interestingly, one of sites that is cleaved by chymotrypsin is residue 682, which is only 2 amino acids farther from a site that was cleaved by trypsin (22). Thus, to further establish the observations presented in Fig. 3, Pgp in
resulting membranes were incubated at 22°C for 5 min in 50 mM Tris-Cl buffer, pH 7.4, containing 0.1 mM Vi, 10 mM MgATP, and 50 μM verapamil (D) as indicated in the figure. Excess ligands in the mixture were removed, and the resulting membranes were assayed for basal (A) and the verapamil-stimulated (B) ATPase activities as described (21). The ATPase activity of these membranes in the absence of any added ligand was considered as 100%. The data represent the mean value of four experiments, and error bars represent the S.D.

Drug Binding to Full-length Pgp—Results presented above suggested that Vi-induced stable inhibition of full-length Pgp at 22°C requires the presence of drug, whereas similar inhibition occurs in the absence of drug at 37°C. To obtain direct evidence for these differences, full-length Pgp was incubated at 22°C or 37°C with various ligands and [125I]IAAP, a photoaffinity analog of prazosin that binds to the drug-binding site of Pgp (27, 35). These preincubation mixtures were exposed to 365 nm of light, and the [125I]IAAP cross-linked Pgp was analyzed by SDS-PAGE followed by fluorography. Fig. 5A shows that at 22°C, Pgp binds [125I]IAAP in the presence of MgATP, MgADP and P0, or MgAMP-PCP. Quantitation of the data from four individual experiments suggested that the amount of [125I]IAAP bound to Pgp is nearly identical in all of the above experimental conditions. These results suggest that, at 22°C, the drug binding site in Pgp is accessible to drug in the presence or absence of nucleotide in the ATP-binding site.

Pgp was also photoaffinity labeled with [125I]IAAP at 37°C in the presence of various ligands, and the results are shown in Fig. 5B. [125I]IAAP was bound to Pgp in the presence of Vi or MgATP, suggesting that the drug-binding site is accessible under these conditions. Interestingly, when both Vi and MgATP were included in the preincubation mixture, the labeling was reduced to ~20%. On the other hand, no significant decrease in the amount of [125I]IAAP bound to Pgp was observed when MgATP was replaced with MgADP and P0, or MgAMP-PCP. These results suggest that, at 37°C, although...
the drug binding site is accessible, conformational changes associated with ATP binding and subsequent hydrolysis in the active site result in occlusion of the drug-binding site. Thus, the difference in the accessibility of the drug-binding site suggests that Pgp assumes two distinct conformational states.

Drug Binding to the Linker Region-cleaved Pgp—The trypsin-cleaved Pgp is a mixture of both the NH2-half (80 kDa) and the COOH-half (60 kDa) of the molecule (21). To understand how cleavage of the linker region affects drug binding, the trypsin-cleaved Pgp was labeled with [125I]IAAP in the presence of Vi and MgATP. Fig. 6 shows that although [125I]IAAP binds to both halves of Pgp in the absence or presence of Vi, the amount of label associated with the COOH-half is higher, corroborating the observations of Ambudkar and his co-workers (27). Interestingly, in the presence of Vi and MgATP, the [125I]IAAP binding to the COOH-half was decreased by ∼50%, without significantly affecting the [125I]IAAP binding to the ∼80 kDa NH2-half. Also as noticed by others (27), an endogenous ∼65 kDa Sf9 insect cell membrane protein is observed as an [125I]IAAP binding protein, which is not reactive with any anti-Pgp antibodies as judged by Western blotting analyses (not shown). Because equal aliquots of the Pgp tryptic digest were used in these incubations, the amount of a given Pgp peptide in each of these reactions is identical. Thus, the decreased [125I]IAAP labeling is likely attributable to the binding of MgATP to ATP binding site resulting in a conformational change that occludes drug binding to a site located in the COOH-half. This occlusion of drug binding is interestingly similar to the drug-binding site occlusion seen in full-length Pgp at 37 °C. Therefore, it is reasonable to suggest that the linker region mediates conformational change in Pgp. Once the linker region is cleaved, the resulting Pgp conformation at 22 °C is similar to the conformation of full-length Pgp observed at 37 °C.

DISCUSSION

A logical approach to understanding the mechanism of active drug transport by Pgp is to first identify its catalytic cycle intermediates. Senior and his colleagues have first, and subsequently others, demonstrated that Vi induces stable inhibition of Pgp ATPase function in the presence of ATP or ADP (23). Importantly, Pgp thus inhibited is relatively a stable complex, which allowed the identification of ligands bound to the Pgp molecule. These studies have identified ADP as the trapped nucleotide in the active site of Pgp (23). The resulting Pgp complex with ADP and Vi, (Pgp.ATP.Vi), was thought to represent a transition state configuration of Pgp in the catalytic cycle (24, 36). Because the transport substrate is an important participant in the transport cycle, without which Pgp does not exhibit ATPase activity (14, 17, 21, 22, 26), we and others have investigated the role of transport substrates in the Vi-induced inhibition of Pgp ATPase process. These studies have interestingly indicated that transport substrate is an essential requirement in the stable inhibition of Pgp ATPase activity (26, 33) and further pointed out that, ATP, the hydrolysable nucleotide substrate of Pgp, is the participating nucleotide in this process (26). On the basis of these requirements in the Vi-induced stable inhibition, we suggested previously that the inhibited Pgp is a complex of ATP, drug, and Vi, (Pgp.ATP.drug.Vi) (26). However, it is not clear how Pgp.ATP.Vi and Pgp.ATP.drug.Vi are formed and whether these represent different intermediates of the catalytic cycle.

Results presented here show the conditions under which the above-mentioned stably inhibited Pgp complexes, Pgp.ATP.Vi and Pgp.ATP.drug.Vi, can be obtained and demonstrate that these represent the intermediates of the catalytic cycle. When Pgp was preincubated with various ligands in the presence of Vi at 22 °C, the drug-stimulated ATPase activity of Pgp could
be stably inhibited only in the presence of ATP and drug (Fig. 1). Absence of ATP, or replacement of ATP in the preincubation mixtures with AMP-PCP, a non-hydrolyzable ATP analog, or with ADP and Pi, the hydrolytic products of ATP, did not result in the inhibition of Pgp ATPase activity, suggesting that the inhibition process is not random but highly specific for the drug and ATP. Senior and his co-workers (23) have shown previously that the nucleotide bound to the stably inhibited Pgp form is ADP, suggesting that the bound ATP likely undergoes hydrolysis. Together these observations suggest that ATP is the only nucleotide to bind to the ATP binding site and undergo hydrolysis, generating the hydrolytic products in the active site itself for a stable inhibition to occur.

Fig. 1 also indicates that Vi-induced stable inhibition of Pgp ATPase activity requires the presence of drug, suggesting that occupancy of the drug-binding site in the Pgp molecule is another essential requirement. To provide direct evidence that the drug-binding site is occupied by the drug, we used [125I]IAAP, a photoaffinity analog of prazosin, because it was firmly shown by others that it binds Pgp with high affinity (27, 35), and such binding can be inhibited competitively by verapamil and cyclosporin A (not shown) (27). The data presented in Fig. 5A show that Pgp binds [125I]IAAP, irrespective of whether or not the combination of ligands in the incubations leads to the stable inhibition of the Pgp ATPase activity in the presence of Vi. These data suggest that, at 22 °C, the drug-binding site in Pgp is available for occupancy and should be occupied for the Vi-induced stable inhibition to occur. These results indicate that, at 22 °C, binding of ATP to the ATP-binding site, and drug to the drug-binding site, are the requisites for the formation of stably inhibited Pgp complex by Vi, and the resulting inhibited species is a complex of Pgp-ATP-drug-Vi.

On the other hand, the only requirement for the Vi-induced stable inhibition of Pgp ATPase at 37 °C is ATP (Fig. 2). Because AMP-PCP or ADP and Pi cannot replace the function of ATP in the stable inhibition process, it is likely, as discussed above, that the binding of ATP to the ATP-binding site, and its subsequent hydrolysis in the active site, are the requisites in the formation of Vi-induced stably inhibited Pgp complex. The [125I]IAAP binding characteristics of Pgp at this temperature show that the drug-binding site is in fact available and occupied by drug, irrespective of the presence of Vi or ATP (Fig. 5B). However, when both of these ligands, i.e. Vi and ATP, are simultaneously bound to Pgp, the drug binding was greatly reduced. This reduction in the drug binding occurs specifically in the presence of ATP, because inclusion of ADP and Pi, or AMP-PCP in the preincubation mixtures did not significantly alter the amount of radioactivity bound to Pgp. Thus, although the drug-binding site in Pgp is available for occupancy at 37 °C, it is likely that hydrolysis of ATP in the ATP-binding site results in a conformational change in the drug-binding site, leading to its occlusion, or reduction in the affinity for drug binding. It is equally possible that hydrolysis of ATP in the active site results in the de-binding (efflux) of drug from the drug-binding site.

Temperature is well known to induce phase transition of lipids in the biomembranes (37). Although the observations made here appear to indicate that temperature is an important parameter influencing the Pgp inhibition process, a closer examination of the data indicates that phase transition of lipids in the biomembranes or any other changes induced by temperature, per se, do not appear to alter the characteristics of Pgp to bind ligands. First, as shown in Fig. 1, Pgp at 22 °C is stably inhibited only in the presence of ATP and drug, and such inhibition at this temperature does not occur when any of these ligands are absent or modified. Second, as shown in Fig. 2, Pgp inhibition takes place in preincubation mixtures that contained ATP and Vi but not in the mixtures containing ADP, ATP and Pi, or AMP-PCP, although all of these preincubations were maintained at 37 °C. Third, the drug-binding site in the Pgp molecule at 37 °C is accessible and is thus occupied by drug in the presence of AMP-PCP or ADP and Pi (Fig. 5B) and appears to be occluded only in the presence of ATP, arguing against the role of temperature in the changes in the drug-binding characteristics. This reasoning also rules out the possibility that an endogenous component in the membrane is made available at 37 °C because of changes in the membrane fluidity at higher temperature that could bind and compete with [125I]IAAP. Importantly, occlusion of drug binding site in the linker region-cleaved Pgp at 22 °C (Fig. 6), which is analogous to the behavior of the full-length Pgp at 37 °C (Fig. 5B), suggests that temperature is not the determining factor in the formation of the two Pgp forms that differ in drug-binding characteristics. Thus, it is likely that Pgp in the Vi-induced stably inhibited complexes obtained at 22 °C and at 37 °C is conformationally different.

Although the above commentary suggests that Pgp exists in at least two different conformations, the use of the linker region-cleaved Pgp in these studies further defines the functional characteristics of these states. As shown previously (21, 22), cleavage of the linker region results in the exhibition of both basal and drug-stimulated ATPase activities. The basal ATPase activity thus generated can be stably inhibited by Vi in the presence of ATP, suggesting that Pgp complex thus formed is analogous to a conformation assumed by the full-length Pgp in the presence of these ligands (ATP and Vi) at 37 °C. Therefore, this conformational state of Pgp mediates the basal ATPase reaction in the catalytic cycle. As shown in Fig. 4, stable inhibition of basal ATPase activity left the drug-stimulated ATPase activity nearly unaffected, suggesting that the drug-stimulated ATPase activity observed subsequent to the inhibition of the basal ATPase activity is attributable to another conformational state of Pgp. This conformational state of Pgp is analogous to the conformational state (observed at 22 °C), requiring binding of drug, ATP, and Vi for the stable inhibition.

Because full-length Pgp that was stably inhibited in either of the above two conformational states failed to exhibit any ATPase activity corresponding to the remaining uninhibited conformational state, it can be inferred that the two Pgp conformations are coupled in the catalytic cycle. Detection of the basal and the drug-stimulated ATPase activities in the linker region-cleaved Pgp suggests that these two conformations of Pgp are uncoupled. Therefore, it is likely that the linker region couples these two conformational states in the catalytic cycle.

In conclusion, these studies show two distinct conformations of Pgp in the catalytic cycle, one mediating the drug-stimulated ATPase activity and the other mediating the basal ATPase activity. These two conformations of Pgp are coupled by the linker region, which conjoins the NH2- and COOH-halves of the Pgp molecule. Interestingly, Sauna and Ambudkar (30) have recently reported the existence of two ATP hydrolytic events in a single Pgp catalytic cycle. Our interpretation that Pgp assumes at least two distinct conformations that exhibit two different ATP hydrolytic events further defines the catalytic cycle.

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