Cross-linking of Human Multidrug Resistance P-glycoprotein by the Substrate, Tris-(2-maleimidoethyl)amine, Is Altered by ATP Hydrolysis

EVIDENCE FOR ROTATION OF A TRANSMEMBRANE HELIX*

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We identified a thiol-reactive substrate, Tris-(2-maleimidoethyl)amine (TMEA), to explore the contribution of the TM segments 6 and 12 of the human multidrug resistance P-glycoprotein (P-gp) during transport. TMEA is a trifunctional maleimide and stimulated the ATPase activity of Cys-less P-gp about 7-fold. Cysteine-scanning mutagenesis of TM12 showed that the activity of mutant V982C was inhibited by TMEA. P-gp mutants containing V982C (TM12) and another cysteine in TM6 were constructed and tested for cross-linking with TMEA. A cross-linked product was observed in SDS-polyacrylamide gel electrophoresis for mutant L339C(TM6)/V982C(TM12). Cross-linking by TMEA also inhibited the ATPase activity of the mutant protein. Substrates such as cyclosporin A, vinblastine, colchicine, or verapamil inhibited cross-linking by TMEA. In the presence of ATP at 37 °C, cross-linking of mutant L339C/V982C was decreased. In contrast, there was enhanced cross-linking of mutant F343C(TM6)/V982C(TM12) in the presence of ATP. These results show that cross-linking must be within the drug-binding domain, that residues L339C(TM6)/V982C(TM12) must be at least 10 Å apart, and that ATP hydrolysis promotes rotation of one or both TM helices.

The human multidrug resistance P-glycoprotein (P-gp) is an ATP-dependent drug pump and has broad substrate specificity in compounds that can be effluxed out of the cell (1, 2). These compounds have diverse structures and include many that are clinically important. Therefore, overexpression of P-gp is often a complicating factor during cancer and AIDS chemotherapy (3–5).

The physiological function of P-gp is unknown. It is normally expressed in many tissues, and the pattern of P-gp expression in tissues and studies on P-gp “knock-out” mice indicate that it may protect the organism from toxins in our environment and in our diet (6–8).

To understand the mechanism of P-gp-mediated drug transport it will be necessary to determine the structure of the protein and to identify the components involved in drug substrate recognition, ATP hydrolysis, and coupling of ATP hydrolysis to drug efflux. P-gp is a member of the ABC (ATP-binding cassette) family of transporters (9, 10). It has 1280 amino acids that are organized as two repeating units of 610 amino acids, which are joined by a linker region of about 60 amino acids (11). In each repeat there are six transmembrane (TM) segments and a hydrophilic domain containing an ATP-binding site (12, 13).

The minimal functional unit in P-gp appears to be a monomer (14). Both nucleotide-binding sites, however, are required for function, because P-gp is inactive when ATP hydrolysis at either site is blocked by mutation or chemical modification (15–20). The nucleotide-binding domains appear to function in an alternating mechanism (21–24).

An important goal in understanding the mechanism of P-gp is to determine the structure of the drug-binding domain and how ATP hydrolysis affects this domain. Photolabeling and mutational studies suggested that the drug-binding domain is in the TM domains (25–35), and the finding that a deletion mutant lacking both nucleotide-binding domains can still bind drug substrates further supports this idea (36). Drug binding also requires the presence of both halves of the TM domains, because drug-stimulated ATPase activity is only observed when both halves are co-expressed (16). This is consistent with recent reports that residues in TMs 4, 5, 6, 10, 11, and 12 must contribute to the drug-binding domain (37–40).

Several studies have shown that TMs 6 and 12 may be particularly important for drug binding (26, 27, 34, 35, 41–44). Residues in these TMs seem to have a higher affinity for MTS-verapamil and dibromobimane. In addition, these TMs are directly connected to their respective nucleotide-binding domains and movement of the nucleotide-binding domains (see Fig. 1) during ATP hydrolysis could cause these TMs to move (45).

In this study we identified a new sulfhydryl-reactive cross-linking agent (TMEA) that is a substrate of P-gp. We used this compound to investigate changes that occur in the TMs during ATP hydrolysis.

EXPERIMENTAL PROCEDURES

Construction of Mutants—Wild-type P-gp has seven endogenous cysteines at positions 137, 431, 717, 956, 1074, 1125, and 1227. None of the cysteines are essential for activity because mutation of all cysteines to alanine (Cys-less P-gp) resulted in an active molecule (12). The Cys-less P-gp cDNA was modified to code for ten histidine residues at the COOH-terminus of P-gp to facilitate expression in E. coli and enable the purification of histidine-tagged protein. P-gp cDNA was modified to code for ten histidine residues at the COOH-terminus of P-gp to facilitate expression in E. coli and enable the purification of histidine-tagged protein.

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1 The abbreviations used are: P-gp, P-glycoprotein; AMP, 5′-adenyl-β,γ-imidodiphosphate; MTS, methanethiosulfonate; TM, transmembrane; TMEA, tris-(2-maleimidoethyl)amine; PAGE, polyacrylamide gel electrophoresis; BMOE, bis-maleimidoethane.
end of the molecule (Cys-less P-gp(His)_{10}). This facilitated purification of the Cys-less P-gp by nickel-chelate chromatography (46). Cysteine residues were then introduced into the Cys-less P-gp(His)_{10} as described previously (39). The integrity of the mutated cDNA was confirmed by sequencing the entire cDNA (47).

Cys-less versions of the NH₂-half (residues 1-682) or COOH-half (residues 682-1280) molecules of P-gp containing the epitope for monoclonal antibody A52 were described previously (48).

Expression and Purification of P-glycoprotein—Expression and purification of histidine-tagged P-gp mutants were described previously (46). Briefly, 50-10 cm diameter culture plates of HEK 293 cells were transfected with the mutant cDNA. After 24 h, the medium was replaced with fresh medium containing 10 μM cyclosporin A. Cyclosporin A is a substrate of P-gp and acts like a powerful chemical chaperone to promote maturation of P-gp (49). The transfected cells were harvested 24 h later and solubilized with 1% (w/v) n-dodecyl-β-d-maltoside, and the mutant P-gp was isolated by nickel-chelate chromatography (nickel-nitrilotriacetic acid columns, Qiagen, Inc., Mississauga, Ontario, Canada).

Measurement of Drug-stimulated ATPase Activity—Mutant P-gp(His)_{10} was eluted from the nickel columns with buffer containing 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 300 mM imidazole (pH 7.0), 0.1% (w/v) n-dodecyl-β-d-maltoside, and 10% (v/v) glycerol and mixed with an equal volume of 10 mg/ml sheep brain phosphatidylethanolamine (Type II-S, Sigma-Aldrich), which was washed and suspended in 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl. The P-gp/lipid mixture was then sonicated for 45 s at 4 °C (bath-type probe, maximum setting, Branson Sonifier 450, Branson Ultrasonic, Danbury, CT). An aliquot of the sonicated P-gp/lipid mixture was assayed for drug-stimulated ATPase activity by addition of an equal volume of buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM MgCl₂, 10 mM ATP, and 2 mM verapamil. The samples were incubated for 30 min at 37 °C, and the amount of inorganic phosphate liberated was determined (50).

For inhibition by TMEA (Pierce, Rockford, IL), the P-gp/lipid mixture was preincubated with TMEA for 10 min at 22 °C. The reaction was stopped by addition of cysteine, pH 7.5, to a final concentration of 40 mM. Drug-stimulated ATPase activity was then determined as described above.

Cross-linking Analysis—Membranes were prepared from transfected HEK 293 cells and suspended in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl). The membranes were then treated with the desired concentration of TMEA for 5–15 min at 22 °C or 37 °C. Reactions were stopped by addition of 2× SDS sample buffer containing 40 mM cysteine. In experiments dealing with the effects of ATP, AMP-PNP, ATP plus vanadate, or drug substrates (cyclosporin A, vinblastine, colchicine or verapamil), the membranes were preincubated with these compounds for 5–10 min at 22 °C before addition of TMEA.

The samples were then subjected to SDS-PAGE on 7.5% acrylamide gels, transferred onto a nitrocellulose sheet, and probed with monoclonal antibody A52 (33) or rabbit polyclonal antibody (14). After reaction with the appropriate horseradish peroxidase-conjugated secondary antibody, the immunoreactive proteins were visualized by enhanced chemiluminescence (Pierce).

To test for the effect of vanadate on cross-linking, orthovanadate was prepared from Na₃VO₄, pH 10 (51), and boiled for 2 min to break down polymeric species. Purified mutant F434C/V982C was reconstituted in 5 mg/ml sheep brain phosphatidylethanolamine and incubated with 0.2 μM vanadate, 5 mM ATP, and 10 mM MgCl₂ for 2 to 20 min at 22 °C or 37 °C. The reaction was stopped by passing the sample through a Centri-Spin 20 column (Princeton Separations, Inc., Adelphia, NJ) that had been equilibrated with Tris-buffered saline buffer, pH 7.4, containing 5 mg/ml sheep brain phosphatidylethanolamine, 5 mM ATP, and 10 mM MgCl₂. The samples were then analyzed by electrophoresis on a 7.5% polyacrylamide gel with 0.1% (v/v) SDS and transferred onto a nitrocellulose sheet, and probed with the appropriate horseradish peroxidase-conjugated secondary antibody (33) or rabbit polyclonal antibody (14). After reac-

RESULTS

TMEA Is a Substrate of P-gp—To determine whether TMs 6 and 12 line the same drug-binding pocket, we searched for the smallest maleimide cross-linker that was a substrate of P-gp. The rationale was that maleimides react specifically with thiols at neutral pH (6.5–7.5) and that a substrate occupying the drug-binding pocket may react with any thiol group that lines the drug-binding pocket. The smallest thiol-specific maleimide cross-linker is bis-maleimidoethane (BMOE) (Pierce) with an 8-Å spacer arm (Fig. 2). To test whether BMOE was a substrate, we assayed for stimulation of the Cys-less P-gp ATPase activity. Binding of substrate to P-gp stimulates its ATPase activity, and there is good correlation between drug-stimulated ATPase activity and drug transport (52). No stimulation of Cys-less P-gp ATPase activity, however, was observed with BMOE concentrations up to 5 mM (data not shown).

A slightly larger maleimide cross-linker is Tris-(2-maleimidomethyl)amine (TMEA) (Pierce), which has a spacer arm of 10.3 Å (Fig. 2). This cross-linker is water-insoluble, has three reactive side chains, and forms non-cleavable thioester linkages. To test whether TMEA was a substrate of P-gp, we assayed for stimulation of Cys-less P-gp ATPase activity. Fig. 3 shows that TMEA stimulated Cys-less P-gp ATPase activity about 7-fold at a concentration of 1 mM with half-maximal stimulation at 310 μM. This indicates that TMEA is a potentially useful thiol-reactive substrate of P-gp that is able to occupy the drug-binding domain.

We then determined if TMEA could interact with TM12, because previous studies with dibromobimane (37) and MTS-verapamil (40) indicated that this TM segment contributes to the drug-binding domain. Each residue in TM12 (residues Val-974 to Phe-994) was mutated to cysteine, and each mutant protein was reacted with 2 mM TMEA for 15 min at 22 °C. After stopping the reaction by addition of cysteine, verapamil-stimulated ATPase activity was determined. A sample that was not...
treated with TMEA served as a control. Verapamil was chosen to measure drug-stimulated ATPase activity, because it is the most potent stimulator of Cys-less P-gp ATPase activity (up to 13-fold) (38). Two mutants, V982C and A985C in TM12 were inhibited 74% and 68%, respectively, by TMEA. The remaining mutants retained greater than 80% of their activity after treatment with TMEA (data not shown). Mutant G989C was not analyzed, because this mutant has very low verapamil-stimulated ATPase activity (10% of Cys-less P-gp) (37).

*Cross-linking of P-gp by TMEA—* Mutant V982C was then selected for further analysis of TMEA interaction with P-gp, because it retains more activity than mutant A985C (82% versus 31%, respectively) (37). We then tested whether a cysteine at position 982 could be cross-linked by TMEA to a cysteine in TM6 (residues 331–351). Mutant P-gps were constructed that contained two cysteines, one at position 982 in TM12 and the other in TM6 (residues 331–351) (Fig. 1). The mutant P-gps were expressed in HEK 293 cells. Membranes were prepared and treated with 1 mM TMEA, and the samples were subjected to SDS-PAGE and immunoblot analysis with anti P-gp antibody. It was previously shown that cross-linked P-gp migrates with slower mobility than uncross-linked P-gp on SDS-PAGE (45, 48, 53). No cross-linked product was observed in Cys-less P-gp or in mutant P-gp with one cysteine (L339C or V982C) (Fig. 4A). In contrast, the presence of a cross-linked product was detected in mutant L339C/V982C (Fig. 4A). The relative amount of cross-linked product increased with increasing concentrations of TMEA (0.3 to 3 mM) (Fig. 4B).

To confirm that TMEA could indeed cross-link L339C(TM6) and V982C(TM12), the half-molecule forms of the P-gp mutants were subjected to cross-linking with TMEA. Each half-molecule P-gp cDNA encodes for either the NH₂- or COOH-terminal half of P-gp. Each half-molecule when expressed alone shows no drug-stimulated ATPase activity. Co-expression of both half-molecules, however, results in association of the polypeptides into a functional transporter that exhibits drug-stimulated ATPase activity (16) and can confer drug resistance to transfected cells (36). Mutations L339C and V982C were then introduced into the Cys-less NH₂ and COOH half-molecules, respectively. The half-molecule mutant cDNAs were then expressed in HEK 293 cells. Membranes were prepared and treated with TMEA. Fig. 5 shows that cross-linking by TMEA occurred only when the L339C(TM6) and V982C(TM12) half-molecules were expressed together. A cross-linked product was not observed when one cysteine was present. These results confirm that TMEA can cross-link L339C and V982C.

**Fig. 3.** Effect of TMEA on Cys-less P-gp ATPase activity. Histidine-tagged Cys-less P-gp was expressed in HEK 293 cells and isolated by nickel-chelate chromatography. The isolated P-gp was mixed with lipid and sonicated, and ATPase activity was determined in the presence of various concentrations of TMEA.

**Fig. 4.** Cross-linking of mutant L339C/V982C. A, membranes prepared from HEK 293 cells expressing Cys-less P-gp and mutants L339C, V982C, or L339C/V982C were treated with (+) or without (−) 1 mM TMEA for 15 min at 22 °C. The reactions were stopped by addition of SDS-sample buffer containing 40 mM cysteine. The samples were subjected to immunoblot analysis followed by enhanced chemiluminescence. B, membranes prepared from HEK 293 cells expressing mutant L339C/V982C were treated with various concentrations of TMEA, and then the samples were subjected to immunoblot analysis as described above. The positions of the cross-linked (X-link) and mature (170 kDa) P-gp are indicated.

**Fig. 5.** Cross-linking of half-molecules of P-gp. Membranes were prepared from HEK 293 cells expressing A52-tagged mutants L339C(N-half)/Cys-less C-half, Cys-less N-Half/V982C(C-half), or L339C(N-half)/V982V(C-half) were treated with 1 mM TMEA for 15 min at 22 °C. The reactions were stopped by addition of SDS-sample buffer containing 40 mM cysteine. The samples were subjected to immunoblot analysis with monoclonal antibody A52 followed by enhanced chemiluminescence. The positions of the cross-linked (X-link), core-glycosylated N-half (N-half(core)), fully glycosylated N-half (N-half(CHO)), and C-half proteins (C-half) are indicated.

**Effect of Drug Substrates on TMEA Cross-linking—** Mutant L339C/V982C was particularly interesting, because previous studies showed that L339C and V982C reacted with sulfhydryl-reactive substrates such as dibromobimane (37) and MTS-verapamil (40). Fig. 6 shows that treatment of mutant L339C/V982C inhibited verapamil-stimulated ATPase activity and that there is fairly good correlation with the concentration required for inhibiting ATPase activity and the appearance of cross-linked product (Fig. 4B). If TMEA is occupying the drug-binding pocket, then other drug substrates should be able to displace it and block cross-linking of the mutant. Cyclosporin A, vinblastine, colchicine, and verapamil were tested for their effect on TMEA cross-linking. P-gp has a very high affinity for cyclosporin A and has been used as an inhibitor of P-gp-mediated drug resistance (54). Verapamil causes the greatest stimulation of P-gp ATPase activity, whereas vinblastine and colchicine have been commonly used to assay for P-gp-mediated drug resistance (2). Mutant L339C/V982C was preincubated with various concentrations of blocking substrates, and then cross-linking was initiated by addition of TMEA (1 mM). Fig. 7 shows that the four drug substrates could block cross-linking with TMEA. Cyclosporin A was the most potent inhibitor of...
cross-linking. At a concentration of 1 \( \mu M \) there was almost complete inhibition of cross-linking. Vinblastine inhibited cross-linking at concentrations of 10 to 100 \( \mu M \), whereas colchicine and verapamil blocked cross-linking at concentrations of 1 mM to 10 mM.

Effect of Nucleotides and Vanadate on Cross-linking—Hydrolysis of ATP can also cause changes in the TM domains of P-gp (45). Therefore, we examined the effects of ATP, AMP-PNP, and ATP plus vanadate on cross-linking of P-gp mutants containing a pair of cysteines with TMEA. The rationale was that a cross-linkable substrate might provide very useful information on nearest neighbor studies and that by having a relatively “fixed” position (V982C(TM12)) it may be possible to determine the potential type of movement of the TM6 during ATP hydrolysis. Vanadate was included in the study because it locks P-gp in an inactive conformation when it occupies one of the nucleotide-binding sites with ADP (55). Fig. 8A shows that vanadate is a potent inhibitor of the ATPase activity of mutant F343C/V982C. More than 80% of the ATPase activity of mutant F343C/V982C was inhibited after treatment for 5 min with vanadate at 22 °C or 37 °C. The activity was almost completely inhibited by vanadate after 10 min. Similar results were obtained with Cys-less P-gp and mutant L339C/V982C (data not shown). The effect of these compounds on cross-linking of mutant L339C/V982C over time with 1 mM TMEA at 37 °C is shown in Fig. 8B. The presence of ATP caused a decrease in the amount of cross-linked product when compared with that without nucleotides. The presence of ATP plus vanadate almost completely inhibited cross-linking of mutant L339C/V982C, whereas the presence of the non-hydrolyzable ATP analog, AMP-PNP, had little effect.

Another mutant, F343C/V982C, showed a quite different pattern of cross-linking with TMEA in the presence of nucleotides. This mutant showed little cross-linking when treated with TMEA at 37 °C in the absence of nucleotides (Fig. 8C, No addition). In the presence of ATP, however, the relative amount of cross-linked product was greatly increased, with a
Rotation of TM Segments of P-glycoprotein

concomitant decrease in the level of the 170-kDa P-gp protein (Fig. 8C, +ATP). It appears that ATP hydrolysis was required to increase the level of cross-linked product, because no enhancement of cross-linking was observed in the presence of AMP-PNP or ATP plus vanadate (Fig. 8C, +AMP-PNP, +ATP/Vo4). These results show that ATP hydrolysis or trapping of vanadate by the nucleotide-binding domains changes the structure of the TM domains. ATP hydrolysis slows down TMEA cross-linking of mutant L339C/V982C while enhancing cross-linking of mutant F343C/V982C.

**DISCUSSION**

TMEA can be added to a list of thiol-reactive compounds such as dibromobimane and MTS-verapamil that appear to be substrates of P-gp because they can stimulate ATPase activity. These compounds, however, have not been shown to be transported by P-gp due to technical problems. Nevertheless, a good correlation between drug-stimulated ATPase activity and drug transport has been shown for vinblastine (52).

In this study we showed that the cross-linker substrate TMEA interacts with TM6 and TM12. The cross-linking was specific, because no cross-linking was detected in mutant L339C/Cys-1074 or F343C/Cys-1074 (data not shown). These mutants were tested as negative controls, because we have observed disulfide cross-linking between Cys-427 or Cys-439 with Cys-1074 (56). An explanation for these results is that TM6 (L339C) and TM12 (V982C) line the same drug-binding pocket. In the absence of ATP, TMEA cross-linking between L339C and V982C was favored, indicating that, during drug binding, P-gp would be in a conformation where L339C and V982C would contribute to the drug-binding pocket. This would be consistent with previous studies with dibromobimane (37) and MTS-verapamil (40) showing that these two residues form part of the drug-binding domain. Both residues (L339C and V982C) reacted with dibromobimane and MTS-verapamil. It is also possible that these residues may not form part of the drug-binding site but are more accessible and reactive to these sulfhydryl-reactive compounds. The evidence suggests, however, that this is quite unlikely. The ability of TMEA to stimulate the ATPase activity of Cys-less indicates that it is likely to be occupying the drug-binding domain and that cross-linking of L339C/V982C would probably not have occurred if these residues were not within or very close to the drug-binding pocket. Also, the concentration of TMEA required to inhibit the ATPase activity of the mutant P-gp appeared to correlate with that required for the detection of cross-linked product (Figs. 4B and 6). Other substrates with diverse structures such as cyclosporin A, vinblastine, colchicine, and verapamil could inhibit cross-linking with TMEA.

There is considerable interest about the number of drug-binding sites in P-gp. There are reports of P-gp having four or more binding sites for drugs and modulators (57–62). Therefore, the question remains whether L339C(TM6) and V982C(TM12) are part of separate drug-binding sites or whether they line the same drug-binding site. Cross-linking of L339C(TM6)/V982C(TM12) by TMEA indicates that both residues participate in the same binding site. This would be consistent with the idea that there is one drug-binding pocket that can accommodate structurally different compounds by contributing different residues in TMs 4, 5, 6, 10, 11, and 12 to the binding of substrate. Slight rotations or twisting of the helices such as those that may occur by introduction of mutations within the helices would result in different residues lining the drug-binding pocket such that it can either alter the affinity of the substrate or accommodate a compound with a different structure. Alternatively, the substrate may induce the proper residues to line the drug-binding pocket (induced-fit model) (38, 63). This can be accomplished because of the relatively mobile nature of the helices (45, 53).

An emerging trend in understanding the mechanism of P-gp drug transport is the role of conformational changes during drug binding and ATP hydrolysis (45, 48, 56, 64–68). ATP hydrolysis appeared to cause TM6, TM12, or both TM segments to undergo a conformational change, because TMEA cross-linking of mutant F343C/V982C was enhanced whereas that of mutant L339C/V982C was decreased. The effect of ATP hydrolysis could be explained if TM6 is modeled as an α-helical wheel. Fig. 9 shows residues 339 and 343 to be on the same face but separated by a turn of the helix. In the absence of ATP, TMEA would react with residues Cys-339 and Cys-982 (Fig. 9A), which are at least 10 Å apart. Upon ATP hydrolysis, a slight rotation of TM6 would make residues Cys-343 more and Cys-339 less accessible, hence increased cross-linking of mutant F343C/V982C (Fig. 9B). It is interesting that ATP-induced conformational changes between TM6 and TM12 had been observed, but it was not clear what type of movement needed to occur during ATP hydrolysis (45). Cross-linking of residues L332C(TM6) and L975C(TM12) was induced by ATP. In the absence of ATP, no cross-linked product was detected in mutant L332C/L975C after TMEA treatment (data not shown) suggesting that these residues might be less reactive with TMEA or that they are more than 10 Å apart. Also, in a helical wheel arrangement, residue Leu-332 is located between Leu-339 and Phe-343, whereas Leu-975 is next to Val-982. Therefore, rotation of TM6 during ATP hydrolysis will result in residue Leu-332 approaching Leu-975 in TM12 for cross-linking by copper phenanthroline (45). It is interesting that cross-linking of mutant L332C/L975C with copper phenanthroline (45) and mutant L339C/V982C with TMEA (this study) was inhibited by ATP plus vanadate. It has been shown that drug transport requires ATP hydrolysis at both nucleotide-binding sites (69). Therefore, cross-linking of F343C/V982C may require ATP hydrolysis at both sites, whereas vanadate trapping at either nucleotide binding site inhibits activity (18). Thus vanadate trapping at one site must “lock” P-gp in a conformation that is not favorable for cross-linking of mutant L339C/V982C or F343C/V982C. Another possibility is that release of ADP may be important for...
a conformation that allows cross-linking, and this is prevented from occurring during vanadate trapping.

This study shows that binding of substrates to P-gp appears to involve some residues that are different than those involved during ATP hydrolysis. Sauna and Ambudkar (61) recently showed that ATP hydrolysis results in a conformation change where the affinity of the substrate, iodoarylazidoprazosin, was dramatically reduced. Based on our results, we propose that movement of the TM segments during ATP hydrolysis through rotation and possible lateral movement of the helices would result in different residues being exposed to the substrate (70). This would be consistent with the idea that drug substrates cause an induced-fit in P-gp such that specific residues from different TMs contribute to the binding of a particular substrate (40). The implication of the results from this study is that rotation of helices during ATP hydrolysis would alter the conformation of the drug-binding site by exposing different residues to the binding site and thereby altering the affinity of P-gp for the bound substrate. This step likely occurs just before the substrate is expelled from the drug-binding pocket. Following ATP hydrolysis and release of substrate, the P-gp molecule would revert to its original conformation to allow the binding of another substrate.

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