Nucleotide Binding, ATP Hydrolysis, and Mutation of the Catalytic Carboxylates of Human P-Glycoprotein Cause Distinct Conformational Changes in the Transmembrane Segments†

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Received May 3, 2007; Revised Manuscript Received June 8, 2007

ABSTRACT: P-Glycoprotein (P-gp, ABCB1) transports a variety of structurally unrelated cytotoxic compounds out of the cell. Each homologous half of P-gp has a transmembrane (TM) domain containing six TM segments and a nucleotide-binding domain (NBD) and is joined by a linker region. It has been postulated that binding of two ATP molecules at the NBD interface to form a "nucleotide sandwich" induces drug efflux by altering packing of the TM segments that make up the drug-binding pocket. To test if ATP binding alone could alter packing of the TM segments, we introduced catalytic carboxylate mutations (E556Q in NBD1 and E1201Q in NBD2) into double-cysteine mutants that exhibited ATP-dependent cross-linking so that the mutants could bind but not hydrolyze ATP. It was found that ATP binding alone could alter disulfide cross-linking between the TM segments. For example, ATP inhibited cross-linking of mutant L339C(TM6)/V982C(TM12)/E556Q(NBD1)/E1201Q(NBD2) but promoted cross-linking of mutant F343C(TM6)/V982C(TM12)/E556Q(NBD1)/E1201Q(NBD2). Cross-linking of some mutants, however, appeared to require ATP hydrolysis as introduction of the catalytic carboxylate mutations into mutant L332C(TM6)/L975C(TM12) inhibited ATP-dependent cross-linking. Cross-linking between cysteines in the TM segments also could be altered via introduction of a single catalytic carboxylate mutation into mutant L332C(TM6)/L975C(TM12) or by using the nonhydrolyzable ATP analogue, AMP·PNP. The results show that the TM segments are quite sensitive to changes within the ATP-binding sites because different conformations could be detected in the presence of ATP, AMP·PNP, during ATP hydrolysis or through mutation of the catalytic carboxylates.

The human multidrug resistance P-glycoprotein (P-gp,[1] ABCB1) is an ATP-dependent drug pump. It transports cytotoxic compounds out of the cell (*1*−*3*). The level of expression of P-gp is relatively high in the apical membranes of epithelial cells of intestine, kidney, liver, and blood−brain/testes barriers (*4*). The physiological role of P-gp is unknown. Mice lacking P-gp can still survive and reproduce but are more sensitive to cytotoxic compounds (*5*).

P-gp is a member of the ATP-binding cassette (ABC) family of transporters (48 human members) (*6*). It has two homologous halves that are joined by a linker region (*7*). Each half has a transmembrane domain (TMD) containing six transmembrane (TM) segments and a nucleotide-binding domain (NBD).

Structural studies on bacterial ABC transporters show that the NBDs have various domains such as the Walker A and B sites, LSGGQ signature motifs, and A-, D-, H-, and Q-loops (*2*, *8*, *9*). A pair of ATP molecules occupy the interface among the Walker A and B sites, H-loop, and Q-loop of one NBD and between the LSGGQ sequence and D-loop of the second NBD. Although there is no high-resolution structure of P-gp, disulfide cross-linking studies have shown that the Walker A sequence in each NBD is close to the opposing LSGGQ signature sequence (*10*).

Drug substrates bind in the drug-binding pocket formed at the interface between the two TMDs (*11*, *12*). A truncated P-gp lacking both NBDs retained the ability to bind drug substrate (*13*). P-gp can simultaneously bind different drug substrates (*14*, *15*), and binding may occur via an "induced-fit" mechanism (*16*).

There is cross-talk between the drug-binding sites and the ATP-binding sites as drug binding can stimulate or inhibit ATPase activity. Drug binding mainly affects the V_{max} for ATP rather than its K_m (*17*, *18*). Drug binding appears to alter the structure of the nucleotide−sandwich dimer (*19*, *20*). Binding of ATP has then been postulated to act as the power stroke in ABC transporters to reorient the drug-binding sites from high-affinity inward-facing orientations to outward-facing low-affinity sites (*21*−*25*). Several studies using ATP analogues have shown that there are alterations in packing of the TM segments (*26*−*29*). We have used cysteine cross-linking to identify ATP-dependent changes in the packing

† This work was supported by a grant from the Canadian Institutes of Health Research. D.M.C. is the recipient of the Canada Research Chair in Membrane Biology.

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[1] Abbreviations: P-gp, P-glycoprotein; M14M, 3,6,9,12-tetraoxatetradecane-1,14-diyl bismethanethiosulfonate; TM, transmembrane; TMD, transmembrane domain containing either the six NH_2- or COOH-terminal transmembrane segments; NBD, nucleotide-binding domain; TMEA, tris(2-maleimidoethyl)amine.

10.1021/bi700837y CCC: $37.00
Published on Web 07/18/2007

of the TM segments of P-gp but did not observe conformational changes in the presence of the nonhydrolyzable ATP analogue, AMP·PNP (*30*, *31*). A potential explanation for the inability to detect conformational changes with AMP·PNP is that the analogue is structurally different from ATP. Recently, it was shown that introduction of the E556Q-(NBD1) and E1201Q(NBD2) mutations into P-gp yielded a mutant that did not hydrolyze ATP but exhibited normal affinity for ATP (*32*).

In this study, we used disulfide cross-linking of mutants containing the catalytic carboxylate mutations to test for conformational changes in the TM segments upon nucleotide binding.

MATERIALS AND METHODS

Construction of Mutants. Construction of mutants containing pairs of cysteines that exhibited ATP-sensitive cross-linking in the presence of copper phenanthroline [L332C-(TM6)/L975C(TM12)] (*33*), 3,6,9,12-tetraoxatetradecane-1,14-diyl bismethanethiosulfonate (M14M) [L339C(TM6)/F728C(TM7)] (*34*), or tris(2-maleimidoethyl)amine (TMEA) [L339C(TM6)/V982C(TM12) and F343C(TM6)/V982C-(TM12)] (*31*) were described previously. Modifications to the P-gp cDNAs to introduce the catalytic carboxylate mutations, E556Q or E1201Q, were performed by oligonucleotide-directed mutagenesis (*35*).

Disulfide Cross-Linking Analysis. The cDNAs of P-gp mutants containing pairs of cysteines were used to transfect 25 plates (10 cm) of HEK 293 cells as described previously (*34*). After 24 h, the medium was replaced and the cells were incubated for an additional 48 h at 27 °C. Membranes were then prepared, and cross-linking was carried out with copper phenanthroline (*30*), M14M (*36*), or TMEA (*31*) as described previously. Samples were subjected to immunoblot analysis using 7.5% (w/v) SDS−PAGE gels and a rabbit polyclonal antibody prepared against NBD2 of P-gp (*37*). The relative amount of cross-linked P-gp was quantitated by first scanning the gel lanes followed by analysis with NIH Image (available at rsb.info.nih.gov.nih-image) and an Apple computer.

Purification of P-gp and Measurement of Drug-Stimulated ATPase Activity. Histidine-tagged P-gp was expressed in HEK 293 cells and isolated by nickel chelate chromatography (*38*). The isolated histidine-tagged P-gp was then mixed with an equal volume of 10 mg/mL sheep brain phosphatidylethanolamine (type II-S, Sigma) that had been washed and suspended in TBS. The P-gp/lipid mixture was then sonicated and ATPase activity measured in the presence of 0.3 mM verapamil. The samples were incubated for 30 min at 37 °C, and the amount of inorganic phosphate released was determined (*39*).

RESULTS

Effect of Catalytic Carboxylate Mutations on Cross-Linking of TM6/TM12 Mutants. A conformational change in the TMDs that is sensitive to ATP binding or hydrolysis is rotation and/or tilting of TM6 relative to TM12 (*31*). The cross-linker tris(2-maleimidoethyl)amine (TMEA) is a P-gp substrate that exhibits ATP-dependent cross-linking of Cys982(TM12) to either Cys339 or Cys343 in TM6 (Figure 1). In the absence of ATP, mutant L339C(TM6)/V982C-(TM12), but not mutant F343C(TM6)/V982C(TM12), was

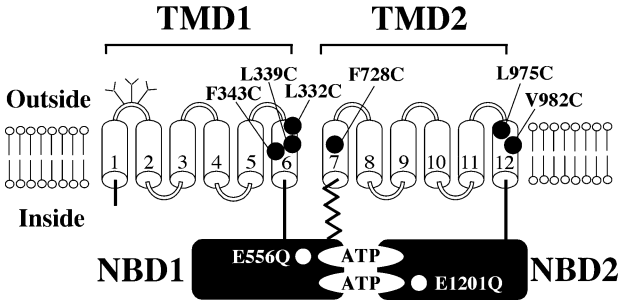

FIGURE 1: Schematic representation of P-gp. The 12 TM segments are depicted as numbered cylinders. The branched lines represent glycosylated sites, and the zigzag lines represent the linker region connecting the two halves of the molecule. There are two ATP-binding sites (ATP) at the interface between the NBDs. The positions of the catalytic carboxylate mutations (E556Q in NBD1 and E1201Q in NBD2) and the cysteine mutations in the TM segments used in the disulfide cross-linking studies (L332C, L339C, F343C, F728C, L975C, and V982C) are shown.

cross-linked with TMEA. In the presence of ATP, however, mutant F343C(TM6)/V982C(TM12), but not mutant L339C-(TM6)/V982C(TM12), was cross-linked with TMEA (*31*). The cross-linking pattern of both mutants with TMEA in the presence of the nonhydrolyzable ATP analogue, AMP·PNP, resembled the results obtained in the absence of nucleotide (*31*). Therefore, it appeared that ATP hydrolysis induced a movement in TM6 to shift cross-linking of Cys982(TM12) from Cys339(TM6) to Cys343(TM6). An alternative explanation was that binding of AMP·PNP did not mimic the movements induced in TM6 by binding of ATP because of their different structures.

To determine whether ATP binding or ATP hydrolysis was responsible for the apparent movement of TM6, we introduced mutations to the catalytic carboxylates into the cross-linkable mutants. Moody et al. (*24*) had reported that mutating the conserved glutamate residues (catalytic carboxylates) within the Walker B sites of the bacterial ABC transporter MJ0796 (from *Methanococcus jannaschii*) resulted in mutants that could bind but not hydrolyze ATP. Similarly, mutation of both Glu556(NBD1) and Glu1201-(NBD2) catalytic carboxylates in human P-gp (Figure 1) to Gln also completely inhibited ATP hydrolysis but had no effect on ATP binding (*32*). Mutation of the catalytic carboxylates of mouse MDR3 to Gln (E552Q/E1197Q) also yields a protein that binds ATP but shows little ATPase activity (reduced more than 400-fold) (*40*). We first determined whether mutation of the catalytic carboxylates in human Cys-less P-gp inhibited ATP hydrolysis. Histidine-tagged Cys-less P-gps containing the E556Q(NBD1) and/or E1201Q(NBD2) mutations were expressed in HEK 293 cells and isolated by nickel chelate chromatography. The isolated mutants were mixed with lipid and assayed for verapamil-stimulated ATPase activity. Verapamil was used because it is a substrate that strongly stimulates Cys-less P-gp ATPase activity (*11*). Figure 2 shows that the presence of either the E556Q(NBD1) or E1201Q(NBD2) mutation reduced verapamil-stimulated ATPase activity to 9.5 or 3.8%, respectively, of that of Cys-less P-gp. Similar results were reported with mouse MDR3 P-gp when either catalytic carboxylate was changed to glutamine (*41*). Verapamil-stimulated ATPase activity was not detected, however, when both catalytic carboxylate mutations were present. These results are in agreement with those of Sauna et al. (*32*), who showed that

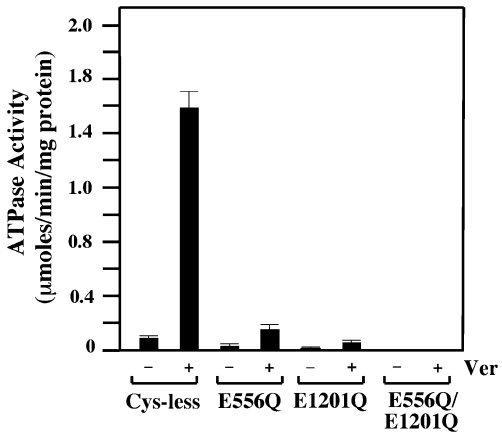

FIGURE 2: Effect of catalytic carboxylate mutations on ATPase activity. HEK 293 cells expressing histidine-tagged Cys-less P-gp (Cys-less) or mutants containing the E556Q(NBD1), E1201Q-(NBD2), or E556Q(NBD1)/E1201Q(NBD2) mutations were isolated by nickel chelate chromatography. The isolated P-gp mutants were mixed with lipid, and ATPase activity was measured in the absence (−) or presence (+) of 0.3 mM verapamil (Ver). Each value is the mean ± the standard deviation (n = 4 separate transfections).

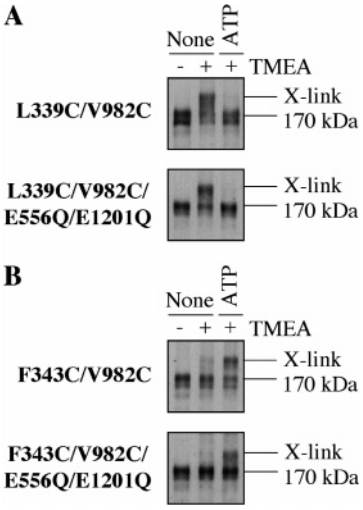

FIGURE 3: Effect of catalytic carboxylate mutations on cross-linking of cysteines in TM6 and TM12 with TMEA. Membranes were prepared from HEK 293 cells expressing P-gp mutants L339C-(TM6)/V982C(TM12) or L339C(TM6)/V982C(TM12)/E556Q-(NBD1)/E1201Q(NBD2) (A) and F343C(TM6)/V982C(TM12) or F343C(TM6)/V982C(TM12)/E556Q(NBD1)/E1201Q(NBD2) (B). The membranes were treated with (+) or without (−) 1 mM TMEA for 15 min at 37 °C in the absence (None) or presence of 5 mM ATP (ATP). The reactions were stopped by addition of SDS sample buffer containing 50 mM EDTA. Samples were subjected to immunoblot analysis with a rabbit polyclonal antibody to P-gp. The positions of mature (170 kDa) and cross-linked (X-link) forms of P-gp are indicated.

the presence of both catalytic carboxylate mutations in wild-type P-gp abolished ATPase activity.

Therefore, the E556Q(NBD1) and E1201Q(NBD2) changes were introduced into mutant L339C(TM6)/V982C(TM12) for cross-linking analysis with TMEA. Cross-linking can readily be detected because introduction of a disulfide linkage between the domains of P-gp causes the protein to migrate with slower mobility in SDS−PAGE gels (*33*). The mutants were expressed in HEK 293 cells. Membranes prepared from the transfected cells were treated with TMEA for 10 min at 37 °C in the presence or absence of ATP. The reactions were stopped by addition of SDS sample buffer containing a thiol reducing agent and samples subjected to immunoblot analysis. Figure 3A shows that introduction of the E556Q(NBD1) and E1201Q(NBD2) mutations into mutant L339C(TM6)/V982C(TM12) did not alter its cross-linking pattern. Cross-linking with TMEA was inhibited by the presence of ATP in both mutants L339C(TM6)/V982C(TM12) and L339C-(TM6)/V982C(TM12)/E556Q(NBD1)/E1201Q(NBD2). These results suggest that ATP binding was sufficient to introduce a conformational change into the TMDs that inhibited cross-linking of Cys339(TM6) to Cys982(TM12) with TMEA.

The E556Q(NBD1) and E1201Q(NBD2) mutations were then introduced into the F343C(TM6)/V982C(TM12) mutant. The mutant was expressed in HEK 293 cells. Membranes were prepared and cross-linked with TMEA in the absence or presence of ATP. The results (Figure 3B) show that mutants F343C(TM6)/V982C(TM12) and F343C(TM6)/V982C(TM12)/E556Q(NBD1)/E1201Q(NBD2) were cross-linked with TMEA only in the presence of ATP. Therefore, ATP binding alone appears to be sufficient for induction of a conformational change in the TMDs to promote cross-linking of Cys343(TM6) to Cys982(TM12) with TMEA.

Effect of Catalytic Carboxylate Mutations on Cross-Linking at the Extracellular Ends of TM6 and -12. Mutant L332C(TM6)/L975(TM12) can be used as a reporter molecule to examine ATP-dependent conformational changes at the predicted extracellular ends of TM6 and -12. Mutant L332C(TM6)/L975C(TM12) contains one cysteine residue at the extracellular end of TM6 (L332C) and another at the extracellular end of TM12 (L975C) (Figure 1). Hydrolysis of ATP promotes cross-linking between Cys332(TM6) and Cys975(TM12) in the presence of oxidant (copper phenanthroline) (*30*).

To test if the catalytic carboxylate mutations would affect ATP-dependent movement of Cys332(TM6) and Cys975-(TM12), the E556Q(NBD1) and E1201Q(NBD2) mutations were introduced into mutant L332C(TM6)/L975C(TM12). The mutants were expressed in HEK 293 cells. Membranes were prepared from transfected cells and then treated with oxidant (copper phenanthroline) in the presence or absence of ATP for 10 min at 37 °C. The reactions were stopped by addition of SDS sample buffer containing 50 mM EDTA but no thiol reducing agent. Samples were subjected to immunoblot analysis. The mutant L332C(TM6)/L975C-(TM12) exhibited extensive cross-linking only in the presence of ATP (Figure 4). By contrast, cross-linked product was not detected in the mutant L332C(TM6)/L975C(TM12)/E556Q(NBD1)/E1201Q(NBD2) in the presence of ATP. Therefore, cross-linking between Cys332(TM6) and Cys975-(TM12) appears to require ATP hydrolysis.

Since cross-linking between Cys332(TM6) and Cys975-(TM12) appeared to require ATP hydrolysis, we would predict that introduction of either catalytic carboxylate mutation should also inhibit cross-linking. Accordingly, mutants Cys332(TM6)/Cys975(TM12)/E556Q(NBD1) and Cys332(TM6)/Cys975(TM12)/E1201Q(NBD2) were constructed. The mutants were expressed in HEK 293 cells, membranes prepared, and samples subjected to cross-linking with an oxidant in the presence or absence of ATP. Figure 4 shows that the presence of the E556Q(NBD1) mutation was sufficient to inhibit cross-linking in the presence of ATP.

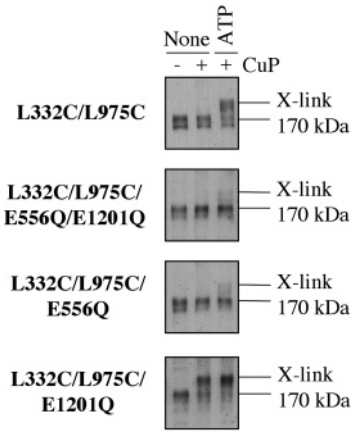

FIGURE 4: Effect of catalytic carboxylate mutations on cross-linking of L332C(TM6)/L975C(TM12). Membranes prepared from HEK 293 cells expressing mutants L332C(TM6)/L975C(TM12), L332C-(TM6)/L975C(TM12)/E556Q(NBD1)/E1201Q(NBD2), L332C-(TM6)/L975C(TM12)/E556Q(NBD1), or L332C(TM6)/L975C-(TM12)/E1201Q(NBD2) were treated with (+) or without (−) 1 mM copper phenanthroline (CuP) for 10 min at 37 °C in the absence (None) or presence of 5 mM ATP (ATP). The reactions were stopped by addition of SDS sample buffer containing 50 mM EDTA and no thiol reducing agent. Samples were subjected to immunoblot analysis using a rabbit polyclonal antibody against P-gp. The positions of mature (170 kDa) and cross-linked (X-link) forms of P-gp are indicated.

Since the E556Q mutation inhibited the verapamil-stimulated ATPase activity of Cys-less P-gp (>90%), the results are consistent with finding that cross-linking between Cys332-(TM6) and Cys975(TM12) requires ATP hydrolysis.

The L332C(TM6)/L975C(TM12)/E1201Q(NBD2) mutant, however, exhibited a quite different pattern of cross-linking. The mutant was cross-linked with oxidant in the presence or absence of ATP (Figure 4). It appeared that the E1201Q-(NBD2) mutation caused a long-range conformational change in the TMDs to allow cross-linking between Cys332(TM6) and Cys975(TM12) in the absence of ATP. The results suggest that the conformation of the TM segments is quite sensitive to changes in the ATP-binding site and that the E556Q and E1201Q mutations induce asymmetric changes in the TMDs. The TMDs appear to undergo different conformational changes during ATP binding, during ATP hydrolysis, or in the presence of the E1201Q mutation.

Effect of ATP on Interactions of Vinblastine with Mutant L339C(TM6)/F728C(TM12). It has been reported that binding of the nonhydrolyzable ATP analogue, AMP·PNP, reduces the affinity of hamster P-gp for vinblastine by ~5−6-fold compared to the presence of ADP (*27*). To test if ATP could also reduce the apparent affinity of P-gp for vinblastine, we used mutant L339C(TM6)/F728C(TM7) (*34*) because its cross-linking is sensitive to the presence of ATP and vinblastine. Figure 5 shows that cross-linking of mutant L339C(TM6)/F728C(TM7) with the M14M cross-linker can be inhibited by vinblastine (Figure 5A) or by vanadate trapping of nucleotide (Figure 5B). Mutant L339C(TM6)/F728C(TM7) is also useful because it can be cross-linked at 0 °C (ice bath) with M14M. At 0 °C, the ATPase activity of P-gp is not detectable (data not shown). Therefore, we first tested whether binding of ATP to P-gp increases the level of vinblastine required to inhibit cross-linking of Cys339(TM6) to Cys728(TM7) without introducing the catalytic carboxylate mutations.

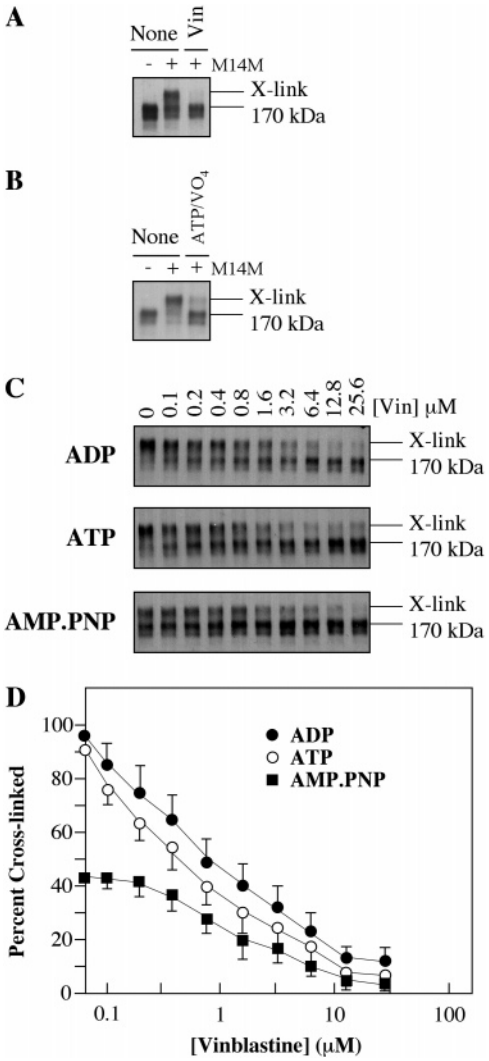

FIGURE 5: Effect of nucleotides and vinblastine on cross-linking of mutant L339C(TM6)/F728C(TM7). (A) Membranes prepared from HEK 293 cells expressing mutant L339C(TM6)/F728C(TM7) were pretreated for 15 min on ice in the absence (None) or presence (Vin) of 0.2 mM vinblastine. Samples were then treated with (+) or without (−) 0.2 mM M14M for 4 min on ice. The reaction was stopped by addition of SDS sample buffer containing 50 mM EDTA and no thiol reducing agent, and the mixture was subjected to immunoblot analysis. (B) For vanadate trapping of nucleotide, the membranes were incubated with 5 mM ATP, 10 mM $MgCl_2$, and 0.2 mM sodium vanadate (ATP/VO4) for 10 min at 37 °C and then chilled on ice. Samples were then treated with (+) or without (−) 0.2 mM M14M for 4 min on ice and the reactions stopped as described above. (C) Membranes prepared from cells expressing mutant L339C(TM6)/F728C(TM7) were incubated on ice in the presence of 5 mM ADP, ATP, or AMP·PNP and various concentrations of vinblastine. Samples were then treated with M14M cross-linker for 4 min on ice. The reactions were stopped by addition of SDS sample buffer containing 50 mM EDTA and no thiol reducing agent. Samples were subjected to immunoblot analysis with a rabbit polyclonal antibody to P-gp. The positions of mature (170 kDa) and cross-linked (X-link) P-gps are indicated. (D) The immunoblots were scanned and quantitated as described in Materials and Methods. The percent cross-linked is the amount of cross-linked P-gp relative to total (cross-linked plus mature P-gps) in the presence of various concentrations of ADP, ATP, or AMP·PNP. Each value is the average ± the standard deviation ($n = 3$).

Accordingly, membranes prepared from HEK 293 cells expressing the mutant L339C(TM6)/F728C(TM7) were pre-incubated for 20 min in an ice bath in the presence of various

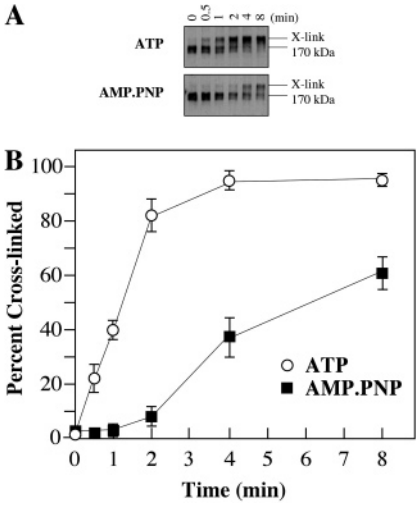

FIGURE 6: Effect of ATP or AMP·PNP on time-dependent cross-linking of mutant L339C(TM6)/F728C(TM7). (A) Membranes prepared from HEK 293 cells expressing mutant L339C(TM6)/F728C(TM7) were incubated on ice for 20 min in the presence of 5 mM ATP or AMP·PNP. Samples were then incubated with 0.2 mM M14M cross-linker for various amounts of time. The reactions were stopped by addition of SDS sample buffer containing 50 mM EDTA and no thiol reducing agent. Samples were then subjected to immunoblot analysis using a rabbit polyclonal antibody against P-gp. The positions of mature (170 kDa) and cross-linked (X-link) forms of P-gp are indicated. (B) The immunoblots were scanned and quantitated as described in Materials and Methods. The percent cross-linked is the amount of cross-linked P-gp relative to total (cross-linked plus mature P-gps) at various times in the presence of ATP or AMP·PNP. Each value is the average ± the standard deviation ($n = 3$).

concentrations of vinblastine and in the presence or absence of 5 mM ATP, ADP, or AMP·PNP. Samples were then treated with 0.2 mM M14M for 4 min on ice. The reactions were stopped by addition of SDS sample buffer containing no reducing agent. Samples were then subjected to immunoblot analysis. Figure 5C shows that in the absence of vinblastine, almost all of the mutant L339C(TM6)/F728C-(TM7) P-gp was cross-linked in the presence of ADP or ATP. Cross-linking was inhibited in a concentration-dependent manner with vinblastine. The concentration of vinblastine required to inhibit cross-linking by 50% in the presence of ATP or ADP was ~500−700 nM (Figure 5D). In contrast, the cross-linking efficiency of mutant L339C(TM6)/F728C-(TM7) in the absence of vinblastine was reduced in the presence of AMP·PNP. In the absence of vinblastine, only ~40% cross-linking of mutant L339C(TM6)/F728C(TM7) was achieved in the presence of AMP·PNP compared to nearly complete cross-linking in the presence of ATP or ADP.

We then compared the kinetics of cross-linking of mutant L339C(TM6)/F728C(TM7) with M14M in the presence of ATP and AMP·PNP. Membranes from cells expressing mutant L339C(TM6)/F728C(TM7) were preincubated with 5 mM ATP or AMP·PNP for 20 min at 0 °C and then treated with 0.2 mM M14M at 0 °C for 0−8 min. The reactions were stopped by addition of SDS sample buffer with no thiol reducing agent, and samples were subjected to immunoblot analysis. Panels A and B of Figure 6 show that 50% cross-linking of the mutant occurred by 1.5 min in the presence of ATP whereas 50% cross-linking in the presence of AMP·PNP occurred at ~6 min. These results show that AMP·PNP alters the kinetics of cross-linking when compared to that with ATP.

An explanation for the observation that the concentration of vinblastine required to protect mutant L339C(TM6)/F728C(TM7) from cross-linking was similar in the presence or absence of ATP (Figure 5C) was that the mutant could still hydrolyze ATP at a very low rate even on ice. To further inhibit the ATPase activity of mutant L339C(TM6)/F728C-(TM7), the E556Q(NBD1) and E1201Q(NBD2) catalytic carboxylate mutations were introduced. The mutant was expressed in HEK 293 cells, and membranes were prepared for cross-linking analysis with the M14M cross-linker in the presence or absence of ATP and various concentrations of vinblastine. Figure 7A shows the inhibition of cross-linking of mutant L339C(TM6)/F728C(TM7)/E556Q(NBD1)/E1201Q(NBD2) by vinblastine in the absence or presence of ATP. In both cases, a 50% decrease in the extent of cross-linking occurred at ~500 nM vinblastine (Figure 7B). It had been previously reported that vinblastine had a higher affinity for P-gp in the absence of nucleotide (*27*). The authors showed that hamster P-gp had a 9-fold difference in the K_d for vinblastine when assayed in the absence of nucleotide or in the presence of AMP·PNP. The results from the hamster P-gp study would predict that the concentration of vinblastine required to inhibit 50% cross-linking of mutant L339C(TM6)/F728C(TM7)/E556Q(NBD1)/E1201Q(NBD2) in the presence of ATP should have been ~4−5 μM.

It is possible that the concentration of vinblastine required to protect mutant L339C(TM6)/F728C(TM7)/E556Q(NBD1)/E1201Q(NBD2) from cross-linking is not different in the presence or absence of ATP because ATP itself may alter interaction of M14M with the mutant. The M14M cross-linker is also a substrate of Cys-less P-gp as it will stimulate the ATPase activity of Cys-less P-gp by 6-fold (*42*). Therefore, we tested whether ATP could alter the concentration-dependent cross-linking of mutant L339C(TM6)/F728C(TM7)/E556Q(NBD1)/E1201Q(NBD2) with M14M. Membranes were prepared from HEK 293 cells expressing mutant L339C(TM6)/F728C(TM7)/E556Q(NBD1)/E1201Q(NBD2) and incubated in the presence of various concentrations of M14M at 0 °C in the presence or absence of ATP. The reactions were stopped by addition of SDS sample buffer containing 50 mM EDTA and no reducing agent. Samples were then subjected to immunoblot analysis. Figure 7C shows that there was little difference in the concentration-dependent cross-linking of mutant L339C-(TM6)/F728C(TM7)/E556Q(NBD1)/E1201Q(NBD2) in the presence or absence of ATP. A level of cross-linking of >50% was observed at 0.2 mM M14M in the presence or absence of ATP (Figure 7D). Therefore, ATP did not appear to affect the apparent affinity of the mutant for M14M.

It was previously observed that incubation of mutant L339C(TM6)/F728C(TM7) in the presence of ATP and sodium orthovanadate inhibited cross-linking with M14M (Figure 5B). Therefore, we confirmed that mutant L339C-(TM6)/F728C(TM7)/E556Q(NBD1)/E1201Q(NBD2) did not exhibit ATP hydrolysis by subjecting it to vanadate trapping of nucleotide. Membranes prepared from cells expressing mutant L339C(TM6)/F728C(TM7)/E556Q(NBD1)/E1201Q-(NBD2) were treated with 5 mM ATP, 10 mM $MgCl_2$, and 0.2 mM sodium vanadate (ATP/VO_4) for 10 min at 37 °C and then chilled on ice. The samples were then treated with

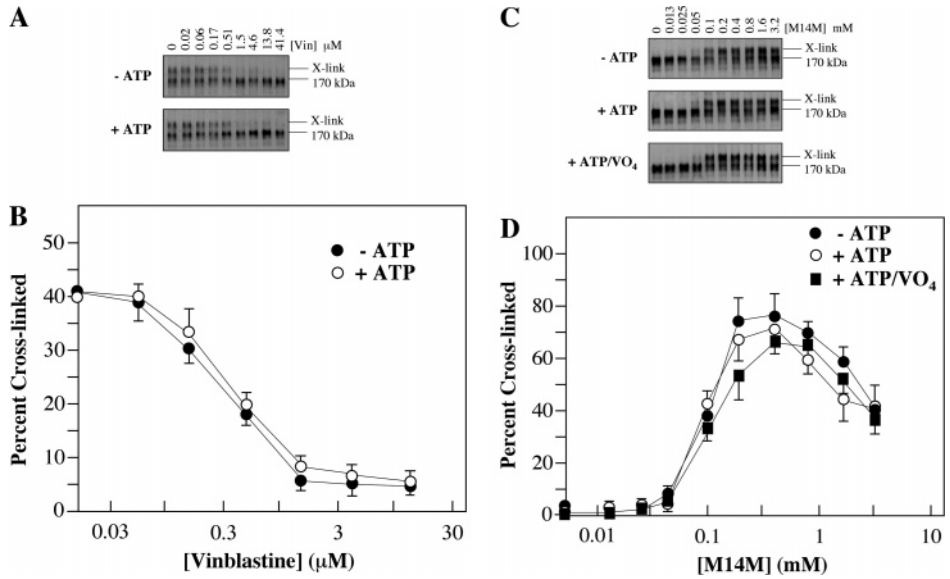

FIGURE 7: Effect of ATP on cross-linking of mutant L339C(TM6)/F728C(TM7) containing the catalytic carboxylate mutations. (A) Membranes were prepared from HEK 293 cells expressing mutant L339C(TM6)/F728C(TM7)/E556Q(NBD1)/E1201Q(NBD2). (A) Samples of membranes were incubated without (−ATP) or with (+ATP) 5 mM ATP in the presence of various concentrations of vinblastine for 15 min on ice. The samples were then treated with 0.2 mM M14M cross-linker for 4 min on ice. The reactions were stopped by addition of SDS sample buffer containing no thiol reducing reagent. Samples were subjected to immunoblot analysis. (B) The immunoblots were scanned and quantitated as described in Materials and Methods. The percent cross-linked is the amount of cross-linked P-gp relative to total (cross-linked plus mature P-gps) in the presence of various concentrations of vinblastine with (+) or without (−) ATP. Each value is the average ± the standard deviation ($n = 3$). (C) Membranes were treated for 15 min on ice in the absence (−ATP) or presence (+ATP) of 5 mM ATP. For vanadate trapping of nucleotide, the membranes were incubated with 5 mM ATP, 10 mM $MgCl_2$, and 0.2 mM sodium vanadate (ATP/VO4) for 10 min at 37 °C and then chilled on ice (ATP/VO_4). The samples were then treated with various concentrations of M14M cross-linker for 4 min on ice. The reactions were stopped by addition of SDS sample buffer containing no thiol reducing reagent. Samples were subjected to immunoblot analysis. The positions of mature (170 kDa) and cross-linked (X-link) P-gps are indicated. (D) The immunoblots were scanned and quantitated as described in Materials and Methods. The percent cross-linked is the amount of cross-linked P-gp relative to total (cross-linked plus mature P-gps) in the presence of various concentrations of M14M cross-linker with (+ATP) or without (−ATP) ATP or after treatment with ATP and vanadate (+ATP/VO_4). Each value is the average ± the standard deviation ($n = 3$).

various concentrations of the M14M cross-linker for 4 min on ice. The reactions were stopped by addition of SDS sample buffer containing no thiol reducing agent and samples subjected to immunoblot analysis. Panels C and D of Figure 7 show that the presence of ATP and vanadate did not inhibit cross-linking with M14M. The results indicate that mutant L339C(TM6)/F728C(TM7)/E556Q(NBD1)/E1201Q(NBD2) did not possess ATPase activity.

DISCUSSION

The results in this study suggest that binding of ATP to the NBDs can induce long-range conformational changes to alter the structure of the TMDs. Binding of ATP appeared to cause a slight rotation or tilting of TM6 so that cross-linking of Cys982(TM12) with TMEA switched from Cys339(TM6) to Cys343(TM6) (Figure 3). Similar effects were observed when the catalytic carboxylates were introduced into the mutants to inhibit ATP hydrolysis (Figure 3). Therefore, ATP binding alone can induce conformational changes in the TMDs (see the model in Figure 8A).

The cross-linking data suggest that TM6 undergoes movement upon binding of ATP, and this observation is in agreement with previous fluorescence studies (*28*). There are differences, however, in the movements induced by ATP and AMP·PNP. For example, the presence of ATP but not AMP·PNP promoted cross-linking of mutant F343C(TM6)/V982(TM12) (*31*). Cross-linking of mutant L339C(TM6)/F728C(TM7) (Figure 6) was observed to be ~4-fold faster in the presence of ATP than in the presence of AMP·PNP. Therefore, binding of ATP or AMP·PNP appears to cause different conformational changes in the TMDs (Figure 8B). It has generally been assumed that ATP and its nonhydrolyzable analogue, AMP·PNP, have similar effects because P-gp has similar affinities for AMP·PNP and ATP (*43*). ATP and AMP·PNP, however, may have different effects on cross-linking because of their different structures. The different pK_a's of their terminal phosphate groups, differences in the P−N and P−O bond lengths, and differences in the P−N−P and P−O−P bond angles may all combine to induce different conformational changes in the TMDs (*44*).

This study and the fluorescence labeling study (*28*) show that nucleotide binding can affect the conformation of TM6. Global changes in the TMDs of P-gp upon nucleotide binding have also been observed by electron microscopy (*45*), protease digestion (*46*, *47*), drug binding (*27*, *48*), photolabeling (*32*), and immunoreactivity (*49*) studies.

The conformational changes in the TMDs appear to be quite sensitive to subtle changes in the ATP-binding sites as the conservative E1201Q mutation had a pronounced effect on the cross-linking pattern of mutant L332C(TM6)/L975C(TM12) (Figure 4). Mutant L332C(TM6)/L975C(TM12) normally exhibits cross-linking only in the presence of ATP. Introduction of the E1201Q(NBD2) mutation, however, enabled the mutant to undergo cross-linking in the absence of ATP. The E1201Q(NBD2) change appears to cause structural changes in the TMDs that mimic some of the movements caused by ATP hydrolysis (Figure 8C). Therefore, the global structure of P-gp appears to be very

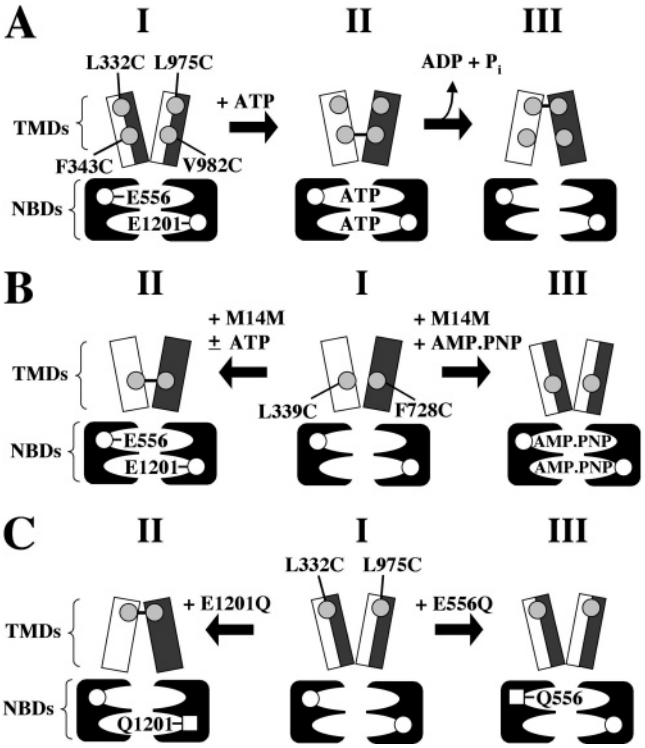

FIGURE 8: Models of TMD conformation changes induced by ATP binding, ATP hydrolysis and catalytic carboxylate mutations. (A) The positions of the cysteine residues [L332C(TM6)/L975C(TM12) and F343C(TM6)/V982C(TM12)] that could be cross-linked and the catalytic carboxylate mutations [E556Q(NBD1) and E1201Q-(NBD2)] are shown. Binding of ATP to P-gp containing the catalytic carboxylates (I) causes conformational changes in the TMDs such that F343C(TM6) and V982C(TM12) could be cross-linked with the TMEA cross-linker (II). Hydrolysis of ATP allows the extracellular end of the TMDs to come close together such that L332C(TM6) and L975C(TM12) could be directly cross-linked in the presence of oxidant, copper phenanthroline (III). It is unknown whether cross-linking between L332C(TM6) and L975C(TM12) is promoted by ATP hydrolysis at one or both ATP binding sites. (B) P-gp mutant L339C(TM6)/F728C(TM7) (I) could be cross-linked with the M14M cross-linker in the presence or absence of ATP (II). In the presence of AMP·PNP, however, there is a decreased level of cross-linking with the M14M cross-linker (III), suggesting that AMP·PNP induces conformational changes in the TMDs that are different from those induced by ATP binding. (C) Mutant L332C(TM6)/L975C(TM12) containing wild-type catalytic carboxylates (I) or the E556Q(NBD1) mutation (III) is not cross-linked by copper phenanthroline. The presence of the E1201Q mutation (II), however, appears to mimic some of the changes induced by ATP hydrolysis since Cys332(TM6) could be cross-linked with Cys975(TM12) with copper phenanthroline.

responsive to events occurring at the ATP-binding sites. The ability of P-gp to show structural alterations in response to small changes in the NBDs suggests that the ATP-binding sites may also operate by an induced-fit mechanism as proposed for the drug-binding sites (*16*). An induced-fit mechanism may explain why P-gp can have similar affinities for ATP and AMP·PNP (*43*), while the F1-ATPase that contains similar ATP-binding motifs (Walker A and B sites) shows an ~100-fold difference in affinity for ATP and AMP·PNP (*50*).

The catalytic carboxylate mutations had asymmetric effects on cross-linking of mutant L332C(TM6)/L975C(TM12) as introduction of the E556Q(NBD1) mutation inhibited cross-linking even in the presence of ATP while the E1201Q-(NBD2) mutation caused cross-linking even in the absence of ATP. It has been previously demonstrated that the ATP sites are not identical (*41*, *47*, *51*−*53*). It has been postulated that binding or hydrolysis of ATP at either site might induce different conformational changes in the protein (*51*). The differential effects of the catalytic carboxylates on cross-linking of mutant L332C(TM6)/L975C(TM12) support their predictions. Since P-gp can simultaneously bind different drug substrates (*14*, *15*), it is possible that ATP binding and/or hydrolysis at each NBD may be coupled to the release of drug substrates from a subset of drug-binding sites. It should be noted, however, that the Gln mutations themselves may have induced asymmetry into the NBDs as suggested by Tombline et al. (*41*). These authors showed that introduction of the mutation E552Q or E1197Q into mouse MDR3 P-gp yielded mutants that exhibited low levels of drug-stimulated ATPase activity, but the E552Q mutant had 3-fold higher activity than the E1197Q mutant. Replacement of the catalytic carboxylates with Ala or Asp, however, yielded mutants that showed similar levels of drug-stimulated ATPase activity. Therefore, only the Gln mutants showed asymmetry. The authors suggested that all of the single-catalytic carboxylate mutants showed some ATPase activity because other residues in the catalytic site could also function as catalytic residues but with reduced efficiency (*41*).

The cross-linking pattern of mutant L332C(TM6)/L975C-(TM12)/E556Q(NBD1)/E1201Q(NBD2) indicates that ATP hydrolysis induces conformational changes in the TMDs that are distinct from those involving ATP binding. The ability to distinguish multiple conformations of P-gp during the catalytic cycle has also been shown by electron cryomicroscopy of hamster P-gp (*26*). Distinct conformations of the protein could be trapped in the absence of nucleotide, in the presence of AMP·PNP, and in the posthydrolytic transition state prior to release of ADP and P_i. Linton and Higgins (*54*) recently postulated that the different conformations of P-gp might have different affinities for drug substrates. Since P-gp has multiple drug-binding sites, it is possible that conversion of different sites from high- to low-affinity states may take place during different stages of the reaction cycle. For example, the authors predicted that ATP binding might be sufficient to reduce the affinity for vinblastine and propafenone but not IAAP. The affinity for IAAP may only show a large reduction in affinity in response to ATP hydrolysis, while the high affinity for propafenone is restored at this step. The high affinity for vinblastine may not be restored until phosphate is released. Therefore, it was proposed that the different drug-binding sites may undergo different major structural rearrangements at the three energetic steps: ATP binding, ATP hydrolysis, and ADP/P_i release.

P-gps from different species show large differences in substrate specificity. For example, rodent P-gps confer stronger resistance to colchicine than human P-gp, whereas human P-gp confers stronger resistance to vinblastine (*55*). Such species differences in substrate specificities could explain why hamster P-gp shows a 9-fold decrease in affinity for vinblastine in the presence of AMP·PNP (*27*). In contrast, we did not observe any detectable difference in the apparent affinity for vinblastine in the presence of ATP (Figures 6 and 7) during cross-linking of mutant L339C(TM6)/F728C-(TM7). It is possible that conversion of the human P-gp vinblastine-binding site to a low-affinity state might occur

at a different energetic state in the reaction cycle such as during ATP hydrolysis. We could detect a conformational change in the mutant L339C(TM6)/F728C(TM12) only upon vanadate trapping of nucleotide (Figure 5B).

In summary, the results of this study support the predictions of the ATP-switch model for the ABC transporter (*54*) in which P-gp undergoes distinct conformational changes upon binding of ATP and during ATP hydrolysis. Conformational changes in the TMDs are sensitive to the state of the ATP-binding sites as conservative changes to the catalytic carboxylates have differential effects on the TMDs and suggest that ATP interaction at either NBD might have different effects of the conformation of the TMDs.

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BI700837Y