Mutation of the Aromatic Amino Acid Interacting with Adenine Moiety of ATP to a Polar Residue Alters the Properties of Multidrug Resistance Protein 1*

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Structural analyses of several bacterial ATP-binding cassette (ABC) transporters indicate that an aromatic amino acid residue in a nucleotide-binding domain (NBD) interacts with the adenine ring of the bound ATP and contributes to the ATP binding. Substitution of this aromatic residue with a polar serine residue in bacterial histidine transporter completely abolished both ATP binding and ATP-dependent histidine transport. However, substitution of the aromatic amino acid residue in the human cystic fibrosis transmembrane conductance regulator with a polar cysteine residue did not have any effect on the ATP-dependent chloride channel function of the protein. To determine whether the other eucaryotic ABC transporters use the strategy analogous to that in some bacterial ABC transporters, the aromatic Trp653 residue in NBD1 and the Tyr1302 residue in NBD2 of human multidrug resistance-associated protein 1 (MRP1) was mutated to either a different aromatic residue or a polar cysteine residue. Substitution of the aromatic residue with a different aromatic amino acid, such as W653Y or Y1302W, did not affect ATP-dependent leukotriene C4 (LTC4) transport. In contrast, substitution of the aromatic residue with a polar cysteine residue, such as W653C or Y1302C, decreased the affinity for ATP, resulting in greatly increased $K_{m}$ values for ATP binding or $K_{p}$ values for ATP in ATP-dependent LTC4 transport. Interestingly, although substitution of the aromatic Trp653 in NBD1 of MRP1 with a polar cysteine residue greatly decreases the affinity for ATP, the ATP-dependent LTC4 transport activities are much higher than that of wild-type MRP1, supporting our hypothesis that the increased release rate of the bound ATP from the mutated NBD1 facilitates the protein to start a new cycle of ATP-dependent solute transport.

Cells overexpressing P-glycoprotein, multidrug resistance-associated protein 1 (MRP1), breast cancer resistant protein, and/or other unidentified drug transporters become resistant to a broad range of anticancer drugs (1–6). These proteins couple ATP binding/hydrolysis to anti-cancer drug transport. Because of the decreased concentration of anti-cancer drugs inside of the cells overexpressing these proteins, the cells become multidrug-resistant. The most conserved features of these proteins are the two cytoplasmic nucleotide-binding domains (NBDs) containing Walker A and B motifs (7); therefore, they belong to the ATP-binding cassette (ABC) transporter superfamily. Recent crystal structural analyses of bacterial ABC transporters, such as HisP (8), MJ0796 (9), MalK (10), and GlcV (11), indicate that: 1) the residues from Walker A motif of one NBD interact with the $\alpha$, $\beta$, and $\gamma$-phosphate of the bound ATP, providing the majority of the interactions that stabilize the nucleotide in the NBD; 2) the Mg$^{2+}$ in the GlcV-ADP-Mg$^{2+}$ complex (11) is bound through an octahedral coordination involving one oxygen atom from $\beta$-phosphate, one oxygen atom from $\gamma$-phosphate, one oxygen atom of Thr$^{165}$ (a residue from Walker A motif), one oxygen atom of Gin$^{166}$ (a residue from Q-loop), two water molecules coordinated with phosphate and Asp$^{165}$ (a residue from Walker B motif), and Glu$^{166}$ (the putative catalytic base); 3) residues from the ABC signature motif of another NBD interact with $\alpha$, $\beta$, and $\gamma$-phosphate, ribose, and adenine base of the bound ATP, further stabilizing the bound nucleotide in the NBD; and 4) additional stabilizing force comes from the interactions between an aromatic amino acid residue and the adenine base of the bound ATP.

Because eucaryotic ABC transporters, such as P-glycoprotein, MRP1, and cystic fibrosis transmembrane conductance regulator (CFTR), also contain Walker A and B motifs, ABC signature motif, and an aromatic amino acid residue; therefore, the way they bind ATP should be analogous to those procaryotic ABC transporters. Mutations of the lysine residue in Walker A motif or aspartic acid residue in Walker B motif in P-glycoprotein (12–16) or in MRP1 (17–20) greatly reduced the protein-mediated ATP-dependent drug transport and drug resistance, supporting the hypothesis that the way to bind ATP by these eucaryotic ABC transporters should be analogous to those procaryotic ABC transporters. Additional evidence comes from the naturally occurring CFTR mutant G551D, a mutation of the Gly$^{551}$ residue in the ABC signature motif of NBD1 causing cystic fibrosis (21–23). Presumably the mutation of this critical Gly residue in the LSGQG ABC signature motif to an acidic amino acid affects ATP binding and the chloride channel function of the protein (24). The corresponding mutations in MRP1, G771D in NBD1 and G1433D in NBD2, almost completely abolished ATP-dependent LTC4 transport (20). However, substitution of the aromatic amino acid with a polar cysteine residue in human CFTR did not disrupt the chloride channel function (25), which was in contrast with the Y16S mutation in bacterial ABC transporter HisP that abolished...
both ATP binding and histidine transport (26). These authors suggested that ATP binding in CFTR may not involve the coordination of the adenine base with an aromatic amino acid residue analogous to that in some bacterial ABC transporters (25). Whether this is due to the edge-to-face interactions between the adenine ring and the aromatic ring of a conserved aromatic residue in the nucleotide binding domain of CFTR remains to be determined. The aromatic residues in the nucleotide binding domain of CFTR are conserved between the bacterial ABC transporters and the mammalian ABC transporters (27), and thus may play a similar role.

**EXPERIMENTAL PROCEDURES**

**Materials**—[8-32P]ATP was purchased from Affinity Labeling Technologies. [14,15,19,20-3H]leukotriene C4 was from PerkinElmer Life Sciences. Grace’s insect cell culture medium was from Invitrogen. Fetal bovine serum was from Gemini Bio-Products. The Stratalinker UV Crosslinker 2400 model (wavelength, 254 nm) and QuikChange Site-Directed Mutagenesis Kit were from Stratagene.

**Cell Culture and Expression of MRPs**—Spodoptera frugiperda Sf21 (Sf21) cells were cultured in Grace’s insect cell medium supplemented with heat-inactivated 5% fetal bovine serum at 27 °C. Viral infection was performed according to Invitrogen’s recommendation.

**Generation of Constructs**—The pDual construct expressing the N-terminal domain of human MRP1 was a gift from Dr. J. R. Riordan. Site-directed mutagenesis of the pDual plasmid was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The forward and reverse primers for the mutation were as follows:

- **Y1302W/forward**, 5'-AGG AAT GCC ACA TTC ACC TAT GCC AGG AGC GAC CCT CCC-3'
- **Y1302W/reverse**, 5'-GAA GTC CAG GTC CTC TCG CCA GCG CAG GCA GTA GTT CCG-3'
- **Y1302C/forward**, 5'-AAT GCC ACA TTC ACC TGT GCC AGG AGC GAC CCT CCC-3'
- **Y1302C/reverse**, 5'-GAA GTC CAG GTC CTC TCG CCA GCG CAG GCA GTA GTT CCG-3'
- **W653Y/forward**, 5'-AGG AAT GCC ACA TTC ACC TAT GCC AGG AGC GAC CCT CCC-3'
- **W653Y/reverse**, 5'-GAA GTC CAG GTC CTC TCG CCA GCG CAG GCA GTA GTT CCG-3'
- **W653C/forward**, 5'-AAT GCC ACA TTC ACC TGT GCC AGG AGC GAC CCT CCC-3'
- **W653C/reverse**, 5'-GAA GTC CAG GTC CTC TCG CCA GCG CAG GCA GTA GTT CCG-3'

**Cell Culture and Expression of MRP1s**—Spodoptera frugiperda Sf21 (Sf21) cells were cultured in Grace’s insect cell medium supplemented with heat-inactivated 5% fetal bovine serum at 27 °C. Viral infection was performed according to Invitrogen’s recommendation.

**Generation of Constructs**—The pDual construct expressing the N-terminal domain of human MRP1 was a gift from Dr. J. R. Riordan. Site-directed mutagenesis of the pDual plasmid was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The forward and reverse primers for the mutation were as follows:

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- **W653Y/forward**, 5'-AGG AAT GCC ACA TTC ACC TAT GCC AGG AGC GAC CCT CCC-3'
- **W653Y/reverse**, 5'-GAA GTC CAG GTC CTC TCG CCA GCG CAG GCA GTA GTT CCG-3'
- **W653C/forward**, 5'-AAT GCC ACA TTC ACC TGT GCC AGG AGC GAC CCT CCC-3'
- **W653C/reverse**, 5'-GAA GTC CAG GTC CTC TCG CCA GCG CAG GCA GTA GTT CCG-3'

**Expression of wild-type and mutant MRP1 proteins in Sf21 insect cells.** A, Mutations of the aromatic residue Trp<sup>653</sup> in NBD1 and Trp<sup>1302</sup> in NBD2 of human MRP1 protein. Boldface letters indicate the residues at position 653 in NBD1 and position 1302 in NBD2. Underlined letters show the mutations. B, expression of wild-type and mutated MRP1 proteins in Sf21 cells. The N-half (933–1531) and C-half (1532–3132) of MRP1 proteins were analyzed by densitometry. The average ratios of wild-type N-half (co-expressed with wild-type C-half) versus 0.4 µg of the W653Y-mutated N-half (co-expressed with wild-type C-half) were determined. Because the ratio of N-half, for example, W653Y-mutated N-half, is similar to that of the wild-type C-half co-expressed with W653Y-mutated N-half, the mean ratio of the protein expression includes N-half and C-half.

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RESULTS

Wild type and Trp<sup>650</sup> and Tyr<sup>1302</sup> Mutants Express Similar Amounts of MRP1 Proteins—Recent crystal structural analyses of bacterial ABC transporters, such as HisP (8), MJ0796 (9), MalK (10), and GlicV (11), indicate that there is an aromatic amino acid residue upstream of the Walker A motif interacting with the adenine ring of the bound ATP and contributing to ATP binding. The aromatic residue Trp<sup>650</sup> in NBD1 of human MRP1 protein interacts with the adenine moiety of the bound ATP (28). The corresponding aromatic amino acid residue in NBD2 of human MRP1 is Tyr<sup>1302</sup>, based on the alignment of the amino acid sequences in that region. To test whether these aromatic amino acid residues are involved in ATP binding, these aromatic residues were mutated to either another aromatic residue, such as Trp or Tyr, or a polar residue, such as Cys, as shown in Fig. 1A. The recombinant viruses expressing wild-type or mutated N-halfs (1–932) and C-halfs (933–1531) were prepared and expressed in Sf21 cells.2 Fig. 1B shows the Western blot results of the proteins expressed in Sf21 cells, probed with antibody 42.4 against NBD1 of human MRP1 protein (19). The expression levels of the dually expressed N-halfs and C-halfs (comparing wild-type C-half with mutated N-halfs) and C-halfs (comparing wild-type C-half with mutated C-halfs) were used to infect Sf21 cells for membrane vesicle preparations.  

MRP1-containing membrane vesicles were prepared according to the procedure described previously (19). Membrane vesicles were resuspended in ice-cold solution containing 10 mM Tris- HCl, pH 7.5, 250 mM sucrose, and 1× protease inhibitors (2 μg/ml aprotimein, 121 μg/ml benzamidine, 3.5 μg/ml E64, 1 μg/ml leupeptin, and 50 μg/ml Pefabloc). After passage through a Liposofast<sup>TM</sup> vesicle extruder (1000-nm nitrocellulose membrane (19)), this nitrocellulose membrane was inoculated in medium containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, and 10 μg/ml tetracyclin. In addition, colonies containing recombinant Bacteria were identified by disruption of the lacZα gene (white colonies) on the LB plates containing 100 μg/ml X-gal and 40 μg/ml isopropyl thiogalactoside was inoculated in medium containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, and 10 μg/ml tetracyclin. The purified recombinant Bacteria were confirmed by polymerase chain reaction with MRP1-specific primers and then used to transfect Sf21 cells with CellFECTIN reagent (Invitrogen). After 3–4 days of incubation at 27 °C, the supernatants containing viral proteins were collected, and the cells lysates (with 2% SDS) were used to do a Western blot, probed with MRP1-specific monoclonal antibodies 42.4 and 897.2 (19).

Viral Plaque Assay, Viral Infection, and Membrane Vesicle Preparation—Viral plaque assay was performed according to Invitrogen’s recommendation. The expression levels of the dually expressed N-halfs and C-halfs with varying multiplicity of infection were determined by Western blot. The multiplicities of infection producing similar amounts of N-halfs (comparing the wild-type N-half with mutated N-halfs) and C-halfs (comparing wild-type C-half with mutated C-halfs) were used to infect Sf21 cells for membrane vesicle preparations.

Membrane Vesicle Transport—ATP-dependent transport of 3<sup>H</sup>-labeled LTEC into the membrane vesicles was assessed by a rapid filtration technique (32, 33). The assays were carried out in a 30-μl solution containing 3 μg of membrane vesicles, 50 mM Tris-HCl, pH 7.5, 2 mM ouabain, 1 mM EGTA, 10 mM MgCl<sub>2</sub>, and varying concentrations of [α-<sup>25</sup>PI]-N<sub>L</sub> for 5 min on ice. The samples were UV-irradiated on ice for 2 min (19, 31), separated on a polyacrylamide gel (7%), and electroblotted to a nitrocellulose membrane.

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Photoaffinity Labeling of MRP1 Protein—Photoaffinity labeling experiments were carried out in a 10 μl of solution containing 5 μg of membrane vesicles, 40 mM Tris-HCl, pH 7.5, 2 mM ouabain, 1 mM EGTA, 10 mM MgCl<sub>2</sub>, and varying concentrations of [α-<sup>32</sup>P]-N<sub>L</sub> for 5 min on ice. The samples were UV-irradiated on ice for 2 min (19, 31), separated on a polyacrylamide gel (7%), and electroblotted to a nitrocellulose membrane.

Membrane Vesicle Transport—ATP-dependent transport of 3<sup>H</sup>-labeled LTEC into the membrane vesicles was assessed by a rapid filtration technique (32, 33). The assays were carried out in a 30-μl solution containing 3 μg of membrane vesicles, 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM MgCl<sub>2</sub>, 200 (see Fig. 2) or 400 mM (see Fig. 3) LTEC, and varying concentrations of ATP as indicated in the figure legends. AMP was used as a negative control. After incubation at 37 °C for the time indicated in the figure legends, the samples were brought back to ice, diluted with 1 μl of ice-cold 1× transport buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 10 mM MgCl<sub>2</sub>) and filtered through a nitrocellulose membrane (19). This nitrocellulose membrane was then washed with 10 μl of ice-cold 1× transport buffer, air-dried, and placed in a 10 μl of biodegradable counting scintillant (Amersham Biosciences). The radioactivity bound to the nitrocellulose membrane was determined by liquid scintillation counting (Beckman LS 6000SC).

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FIG. 2. LTEC uptake by membrane vesicles containing wild-type and mutated MRP1 proteins in the presence of 50 μM ATP. Membrane vesicle preparation and LTEC transport experiments were performed according to the methods described under “Experimental Procedures.” The membrane vesicles containing the same amount of MRP1 proteins (adjusted according to the relative expression levels in Fig. 1) were used to perform LTEC transport experiments in the presence of 50 μM ATP and 200 μM LTEC at 37 °C for 4 min. Amino acid residues at positions 653 and 1302 are indicated at the top of each column. The results shown are the means ± S.D. of triplicate determinations in a single experiment.

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FIG. 3. ATP-dependent LTC4 transport by membrane vesicles containing wild-type and mutant MRP1 proteins. LTC4 transport experiments were performed in a 30-μl solution containing 400 nM LTC4 (12.64 nCi of 3H-labeled LTC4) and varying concentrations of ATP as indicated in the figures at 37 °C for 1 min. The radioactivity bound to the nitrocellulose membranes (3H-labeled LTC4 transported into membrane vesicles) was determined by liquid scintillation counting. The data shown are the means ± S.D. of triplicate determinations in at least three independent experiments. A and B, ATP-dependent LTC4 transport by membrane vesicles containing wild-type and mutant MRP1 proteins with aromatic residues at both positions 653 and 1302, plotted out in 0–4000 μM ATP (A) and 0–500 μM ATP (B). C and D, ATP-dependent LTC4 transport by membrane vesicles containing wild-type and mutant MRP1 proteins with a cysteine residue at position 653. E and F, ATP-dependent LTC4 transport by membrane vesicles containing wild-type and mutant MRP1 proteins with a cysteine residue at position 1302. The transport profile of the wild-type N-half + wild-type C-half is indicated by a dashed line.
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The mean $K_m$ (μM ATP) and $V_{max}$ (pmol of LTC4/mg of protein/min) of wild-type and mutant MRP1s

| Protein          | Amino acid at position | $K_m$ (μM ATP) | $V_{max}$ (pmol of LTC4/mg of protein/min) |
|------------------|------------------------|----------------|------------------------------------------|
| Wild-type MRP1   | Trp                     | 69.0 ± 5.2     | 389.0 ± 32.9                             |
| W653Y            | Tyr                     | 46.5 ± 0.7     | 820.0 ± 21.2                             |
| Y1302W           | Trp                     | 47.7 ± 2.1     | 386.7 ± 60.3                             |
| W653Y/Y1302W     | Tyr                     | 65.5 ± 0.7     | 499.0 ± 5.66                             |
| W653C            | Cys                     | 311.7 ± 46.5   | 881.7 ± 78.5                             |
| W653C/Y1302W     | Cys                     | 290.0 ± 10.0   | 1353.3 ± 203.4                           |
| Y1302C           | Tyr                     | 340.0 ± 42.4   | 700.0 ± 70.7                             |
| W653Y/Y1302C     | Tyr                     | 395.0 ± 15.0   | 380.3 ± 66.9                             |
| W653C/Y1302C     | Cys                     | 1573.3 ± 25.2  | 782.7 ± 20.5                             |

* The $K_m$ (n = 3) and $V_{max}$ (n = 3) values were derived from Fig. 3.

The greatly increased $K_m$ (ATP) values of those mutants containing a polar residue imply that the substitution of the aromatic residue with a nucleophilic cysteine residue decreases the affinity for ATP. However, because the $K_m$ (ATP) value reflects the total events of ATP binding/hydrolysis at both NBD1 and NBD2 and the ATP-dependent LTC4 transport, it might be difficult to make that conclusion based solely on the $K_m$ (ATP) values. To test whether these substitutions really alter their affinities for ATP, membrane vesicles containing wild-type N-half (Trp653) and wild-type C-half (Tyr1302), W653C-mutated N-half and Y1302W-mutated C-half, wild-type N-half and Y1302W-mutated C-half, wild-type C-half, W653Y-mutated N-half and Y1302W-mutated C-half, or W653C-mutated N-half and Y1302C-mutated C-half were labeled with [α-32P]8-N3ATP on ice to determine their $K_m$ (ATP) values (Fig. 4). Because the labeling reactions were performed on ice, ATP hydrolysis reaction should be greatly reduced, and the labeling level should mainly reflect the equilibrium of forward and backward reactions:

$$ V_f = \frac{k_1 [ATP][NBD]}{K_d + [ATP][NBD]} $$

where the velocity of forward reaction $V_f = k_1 [ATP][NBD]$, and the velocity of the backward reaction (or releasing) $V_b = k_{-1} [ATP][NBD]$. At the equilibrium, $V_f = V_b$; therefore, $k_1 [ATP][NBD] = k_{-1} [ATP][NBD]$ and $K_d = k_1/k_{-1} = [ATP][NBD]/[ATP][NBD]$. A higher $K_d$ value for a mutated NBD indicates a decreased affinity for ATP of that specifically mutated NBD.

_panels A, D, G, and J of Fig. 4 show the autoradiograms reflecting [α-32P]8-N3ATP labeling of the wild-type and mutated N- and C-halves on ice. Labeling was determined by Packard Instant Imager and plotted out against [α-32P]8-N3ATP concentrations (Fig. 4, B, C, E, F, H, I, K, and L). The $K_d$ for wild-type NBD1 co-expressed with wild-type NBD2 is ~9 μM ATP, whereas the $K_d$ for wild-type NBD2 co-expressed with wild-type NBD1 is ~33 μM ATP (Table II), which are similar to the $K_d$ values of NBD1 and NBD2 determined from the full-length MRP1 protein (35). In contrast, the $K_d$ value for W653C-mutated NBD1, co-expressed with Y1302W-mutated NBD2, could not be determined because of very weak labeling of this mutated fragment (Fig. 4D), presumably with a very high $K_d$ value. Interestingly, the $K_d$ value for Y1302W-mutated NBD2, co-expressed with W653C-mutated NBD1, increased from 33 (the $K_d$ of wild-type NBD2) to 139 μM ATP (Table II), presumably because of the negative effect of W653C-mutated NBD1 on the Y1302W-mutated NBD2. Therefore there might be an allosteric interaction between the two NBDs even though the experiments were performed on ice. The very weak labeling of W653C-mutated NBD1, including the labeling of W653C-mutated NBD1 co-expressed with Y1302W-mutated (Fig. 4D) and
Y1302C-mutated (Fig. 4J) NBD2, indicates that substitution of the aromatic residue with a polar amino acid greatly decreases the affinity for ATP at this mutated NBD1. The $K_d$ value of W653Y-mutated NBD1, co-expressed with Y1302C-mutated NBD2, increased from 9 (the $K_d$ of wild-type NBD1) to 29 (Table II), presumably because of the negative effect of Y1302C-mutated NBD2. The $K_d$ values of the Y1302C-mutated NBD2 (Table II) increased from 33 (the $K_d$ of wild-type NBD2) to 122 (the $K_d$ of Y1302C-mutated NBD2 co-expressed with W653C-mutated NBD1) and 160 μM ATP (the $K_d$ of Y1302C-mutated NBD2 co-expressed with W653C-mutated NBD1), indicating that substitution of this aromatic residue with a polar amino acid also decreased the affinity for ATP at the mutated NBD2. The combinations of the decreased affinities for ATP at
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| Protein            | Amino acid at position | $K_D$ at NBD1$^a$ | $K_D$ at NBD2$^b$ |
|--------------------|------------------------|------------------|------------------|
| Wild-type MRP1     | Trp                     | 8.7 ± 1.5        | 32.7 ± 3.5       |
| W653C/Y1302W       | Cys                     | ND$^b$           | 139.3 ± 11.0     |
| W653Y/Y1302C       | Tyr                     | 28.7 ± 1.5       | 121.7 ± 7.6      |
| W653C/Y1302C       | Cys                     | ND                | 159.7 ± 31.2     |

$^a$ The $K_D$ values (n = 3) were derived from Fig. 4. $^b$ Not determined because of the very weak labeling of this fragment.

The above conclusions make it possible to determine whether the aromatic residue Trp$^{653}$ in NBD1 and/or Tyr$^{1302}$ in NBD2 of human MRP1 protein are/is involved in nucleotide binding. The approach utilized is to replace the aromatic residues at those two positions with either a different aromatic residue or a moderately polar amino acid. Fig. 1B shows that all of these mutants, no matter whether they were mutated to a different aromatic residue or a polar cysteine residue, produced similar amounts of MRP1 protein as wild type, implying that the substitution with a moderately polar amino acid does not have a significant effect on protein processing and stability. Figs. 2 and 3 show that all of these mutants have the ability to transport LTC4, indicating that these mutations, including the one with a polar residue replacement, do not induce a significant global conformational change and have the ability to bind and hydrolyze ATP.

The above conclusions make it possible to determine whether these aromatic residues in NBD1 and NBD2 are involved in ATP binding. Substitution of the aromatic residue with a different aromatic amino acid do not have a significant negative effect on their ATP-dependent LTC4 transport (Figs. 2 and 3). Their Michaelis constant $K_m$ (ATP) values in ATP-dependent LTC4 transport are similar to that of wild type (Table I), implying that these mutations with a different aromatic residue do not significantly change their affinities for ATP.

TABLE II

**The mean $K_D$ ($\mu$M ATP) of wild-type and mutant MRPs**

| Protein            | Amino acid at position | $K_D$ at NBD1$^a$ | $K_D$ at NBD2$^b$ |
|--------------------|------------------------|------------------|------------------|
| Wild-type MRP1     | Trp                     | 8.7 ± 1.5        | 32.7 ± 3.5       |
| W653C/Y1302W       | Cys                     | ND$^b$           | 139.3 ± 11.0     |
| W653Y/Y1302C       | Tyr                     | 28.7 ± 1.5       | 121.7 ± 7.6      |
| W653C/Y1302C       | Cys                     | ND                | 159.7 ± 31.2     |

$^a$ The $K_D$ values (n = 3) were derived from Fig. 4. $^b$ Not determined because of the very weak labeling of this fragment.

In contrast, the substitutions of the aromatic Trp$^{653}$ and/or Tyr$^{1302}$ with a polar cysteine residue greatly increased their Michaelis constant $K_m$ (ATP) values (Table I), implying that these mutations with a different aromatic residue decreased its affinity for ATP, resulting in increased $K_m$ (ATP) values in ATP-dependent LTC4 transport (Table I) and $K_D$ values in ATP binding (Table II). Whether this decreased affinity for nucleotide in NBD2 also facilitates the molecule to start a new cycle of ATP-dependent solute transport is not clear, because the Y1302C-mutated NBD2 co-expressed with wild-type NBD1 resets the MRP1 protein back to its original conformational state so that the molecule can start a new cycle of ATP-dependent solute transport, leading to a higher $V_{max}$ (LTC4) value than that of wild-type MRP1.

The substitution of the aromatic residue Tyr$^{1302}$ in NBD2 with a polar amino acid residue also decreased its affinity for ATP, resulting in increased $K_m$ (ATP) values in ATP-dependent LTC4 transport (Table I) and $K_D$ values in ATP binding (Table II). Whether this decreased affinity for nucleotide in NBD2 also facilitates the molecule to start a new cycle of ATP-dependent solute transport is not clear, because the Y1302C-mutated NBD2 co-expressed with wild-type NBD1 increased its $V_{max}$ (LTC4) 1.8-fold (Table I), whereas the Y1302C-mutated NBD2 co-expressed with W653Y-mutated NBD1 did not have a significant effect on its $V_{max}$ (LTC4) value (Table I).

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