ATP Binding to the First Nucleotide Binding Domain of Multidrug Resistance-associated Protein Plays a Regulatory Role at Low Nucleotide Concentration, whereas ATP Hydrolysis at the Second Plays a Dominant Role in ATP-dependent Leukotriene C4 Transport*

Runying Yang, Liying Cui, Yue-xian Hou, John R. Riordan, and Xiu-bao Chang†

From the Mayo Foundation, S. C. Johnson Medical Research Center, Mayo Clinic Scottsdale, Scottsdale, Arizona 85259

Multidrug resistance-associated protein (MRP1) transports solutes in an ATP dependent manner by utilizing its two nonequivalent nucleotide binding domains (NBDs) to bind and hydrolyze ATP. The two NBDs possess different properties (Gao, M., Cui, H. R., Loe, D. W., Grant, C. E., Almquist, K. C., Cole, S. P., and Deeley, R. G. (2000) J. Biol. Chem. 275, 13098–13108; Hou, Y., Cui, L., Riordan, J. R., and Chang, X. (2000) J. Biol. Chem. 275, 29280–29287) and may play different roles during solute transport. We now report that NBD1 has moderately higher affinity for ATP than NBD2. The consequence of this difference is that the overall $K_a$ value for wild-type MRP1 is mainly determined by ATP binding at NBD1. This conclusion is supported by the following: 1) mutation of the cysteine residue at 682 to alanine (C682A) in Walker A motif decreases the $K_a$ value, indicating increased affinity for ATP; 2) mutation of the alanine residue at 1331 to cysteine (A1331C) in the Walker A motif of NBD2 does not have an effect on the $K_a$ value; and 3) photolabeling of the protein with a cysteine residue in the Walker A motif of NBD1 is much more sensitive to N-ethylmaleimide modification than the protein with a cysteine residue in the Walker A motif of NBD2. In contrast, the $K_m$ for ATP in support of LTC4 transport is mainly determined by ATP hydrolysis at NBD2. This conclusion is supported by the following: 1) although mutation of A1331C does not have an effect on the $K_m$ value, the $K_m$ values measured from LTC4 transport by proteins with this mutation in NBD2 are much higher than the proteins with wild-type NBD2, implying that the A1331C mutation affects ATP binding/hydrolysis at NBD2; and 2) ATP-dependent LTC4 transport by the protein with a cysteine residue in the Walker A motif of NBD2 is much more sensitive to N-ethylmaleimide modification than the protein with a cysteine residue in the Walker A motif of NBD1. Our previous results indicated that ATP binding at NBD1 at low concentration enhanced ATP binding/hydrolysis at NBD2. All of these results support the notion that ATP binding at NBD1 at low concentration plays a more important regulatory role than the binding at high ATP concentration and that ATP hydrolysis at NBD2 plays a dominant role in the ATP-dependent LTC4 transport.

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†To whom correspondence should be addressed: S. C. Johnson Medical Research Center, Mayo Clinic Scottsdale, 15400 E. Shea Blvd., Scottsdale, AZ 85259. Tel.: 480-301-6206; Fax: 480-301-7017; E-mail: xbchang@mayo.edu.

It is widely accepted that overexpression of P-glycoprotein (P-gp)$^1$ and/or multidrug resistance-associated protein (MRP1) confers cancer cells resistance to a broad range of anticancer drugs. Although they pump solutes out of cells in different fashions (i.e. P-gp transports hydrophobic compounds directly (1–3), whereas MRP1 transports anionic conjugates, such as glutathione-, glucuronide-, or sulfate-conjugated alphatic, prostanoit, or heterocyclic compounds) (4–12), both of them transport anticancer drugs out of cells in an ATP-dependent manner by utilizing their membrane-spanning domains and two nucleotide binding domains (NBDs) (13–15) (i.e. they couple ATP binding and hydrolysis to transport of solutes) (4–12, 16). However, it seems unlikely that they share the same mechanism of this coupling. First, the two NBDs of P-gp have been shown to be functionally equivalent, with identical ATP hydrolysis steps occurring alternately at each NBD (17–22). In other reports, the two NBDs of P-gp were found to be essential for its function but not entirely symmetric (23, 24). It has been clearly demonstrated that the two NBDs of MRP1 have different properties and functions based on mutations, ATP binding, and ATP-dependent LTC4 transport (25–28). Ambudkar's group reported that there are two independent ATP hydrolysis events in a single drug transport cycle by P-gp (29); one ATP hydrolysis is associated with drug transport, whereas the other causes conformational resetting to the original state of the molecule (30). However, in these interpretations, the two NBD sites of P-gp are recruited in a random manner with equal affinity for ATP binding (30). The two ATP binding/hydrolysis sites of MRP1 seem not to be recruited in a random manner, because photolabeling by the nonhydrolyzable [α-32P]8-N3ATP or [γ-32P]8-N3AMP-PNP, which, in the absence of other nucleotides, occurred predominately at NBD1, was enhanced at NBD2 by a low concentration of 8-N3ATP, implying that NBD1 might have higher affinity for nucleotide binding than NBD2 (31). A similar conclusion can be reached from experiments in which the N-proximal half and C-proximal half MRP1 were expressed simultaneously in sf21 cells and labeled with either [α-32P]8-N3-ATP or [γ-32P]8-N3ATP on ice (25). However, experiments to date have not directly compared the affinity of the two NBDs for nucleotides. We have now done this and found that NBD1 of MRP1 protein has higher affinity for N3-ATP than NBD2. The differences between the two NBDs should reflect the different

$^1$The abbreviations used are: P-gp, P-glycoprotein; MRP1, multidrug resistance protein; NBD, nucleotide binding domain; TPCK, L-1-tosyl-amido-2-phenylethyl chloromethyl ketone; 8-N3ATP, 8-azidoadenosine 5′-triphosphate; LTC4, leukotriene C4; BHK, baby hamster kidney; NEM, N-ethylmaleimide; ABC, ATP-binding cassette.
structures of these domains, especially the amino acid residues involved in ATP binding, such as Walker A and B motifs and ATP binding cassette (ABC) signature sequences (32–34). We noticed that the cysteine residue was conserved in Walker A motifs in NBD1 and NBD2 of P-gp, whereas it was not conserved in the NBD2 Walker A motif of MRP1 (Fig. 1A). Therefore, we switched these residues between NBD1 and NBD2 to study the properties of these two mutated NBDs. After switching these residues, we found that affinities for ATP binding were changed as were sensitivities to Walker A and B triplicate determinations.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sodium orthovanadate, NEM, EGTA, EDTA, ATP, ouabain, and TPCK-treated trypsin were purchased from Sigma. \( ^{32} \)P-labeled ATP was purchased from Affinity Labeling Technologies. [14,15,19,20-\(^{3} \)H]leukotriene C4 was from PerkinElmer Life Sciences. Dulbecco’s modified Eagle’s medium/F-12 cell culture medium was from Gibco. The Stratagene UV cross-linker 2400 model (wavelength 254 nm) and QuikChange site-directed mutagenesis kit were from Stratagene. Chemiluminescent substrates were from Pierce.

**Cell Lines Expressing MRP1s in BHK Cells and Cell Culture**—Baby hamster kidney (BHK-21) cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 5% fetal bovine serum at 37 °C in 5% CO2. A stable cell line expressing wild-type MRP1 was established previously (35). Stable cell lines expressing mutants C682A, A1331C, and C682A/A1331C were established by selecting the transfected BHK cells with 200 µM methotrexate. Cells for membrane vesicle preparations were grown in roller bottles (BELLCO) in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 5% fetal bovine serum at 37 °C.

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**In Vitro Mutagenesis of MRP1 cDNA to Generate C682A, A1331C, and C682A/A1331C**—The oligonucleotides to introduce the mutations in MRP1 are as follows: MRP/C682A-forward, 5'-GGTGGGCGAGGTG-GGCCCGGAAAATGCTGCTCCCTGC-3'; MRP/C682A/reverse, 5'-GCCAGGGACATTTGCCGGCCACCTGGCCGAC-3'; MRP/A1331C-forward, 5'-ATCGTGGGCGGACGGAATGTTGAAGGCTGCTCCCTGCACC-3'; and MRP/A1331C/reverse, 5'-GGTGCGGGAGGAGCTTCCAC-
The membrane vesicles were resuspended in an ice-cold solution containing 10 mg of Mrp1 membrane vesicles and varying concentrations of [3H]-labeled LTC4. The assays were carried out in triplicate. The mean Km and Vmax values (n = 2) were derived from at least 3 experiments. 

TABLE I

| Protein          | Km (µM ATP) | Vmax (pmol of LTC4/mg of protein/min) |
|------------------|-------------|--------------------------------------|
| Wild-type Mrp1   | 29.5 ± 2.3  | 229.7 ± 17.6                         |
| C682A            | 8.5 ± 1.5   | 272.2 ± 0.8                          |
| A1331C           | 17.0 ± 1.0  | 222.3 ± 1.1                          |
| C682A/A1331C     | 9.0 ± 1.0   | 181.8 ± 3.2                          |

The Km values (n = 2) and Vmax values (n = 2) were derived from Fig. 3.

ATCCCGTCGCCCGCCACGAT-3'. The underlined sequences are mutated nucleotides. The coding sequence of human Mrp1 cDNA in the pNUT expression vector (35) was used as template for the mutagenesis. The cysteine residue at position of 682 was mutated to alanine (Fig. 1B, C682A) by using the forward/reverse primers and the quickchange site-directed mutagenesis kit from Stratagene (26). A1331C was also introduced into the cDNA by the same strategy. The BamHI (1036)-EcoRI (4076) fragment containing the C682A mutation and the EcoRI (4076)-SrfI (4520) fragment covering A1331C mutation were sequenced completely and confirmed the mutations. In order to ensure that no other mutations were introduced into the cDNA during the mutagenesis, the BamHI (1036)-EcoRI (4076) fragment in pNUT-MRP/His (35) was replaced by the counterpart fragment containing C682A to generate pNUT-MRP/C682A/His. The EcoRI (4076)-SrfI (4520) fragment in pNUT-MRP/His (35) was replaced by the counterpart fragment containing A1331C to generate pNUT-MRP/A1331C/His. The BamHI (1036)-EcoRI (4076) fragment and the EcoRI (4076)-SrfI (4520) fragment in pNUT-MRP/His (35) were replaced by the counterpart fragments containing C682A and A1331C to generate pNUT-MRP/C682A/A1331C/His. 

SDS-PAGE and Western Blot—SDS-PAGE and Western blot were performed as described previously (26, 27). The primary antibody used was a mouse anti-human Mrp1 monoclonal antibody 42.4 (26, 27), and the secondary antibody was anti-mouse Ig conjugated with horseradish peroxidase. Chemiluminescent film detection was performed according to the manufacturer’s recommendations. 

Membrane Vesicle Preparations—Mrp1-containing membrane vesicles were prepared according to the procedure described previously (26). The membrane vesicles were resuspended in an ice-cold solution containing 10 mm Tris-HCl, pH 7.5, 250 mm sucrose, and 1× protease inhibitors (2 µg/ml aprotinin, 121 µg/ml benzamidin, 3.5 µg/ml E64, 1 µg/ml leupeptin, and 50 µg/ml Pefabloc). After passage through a Liposafe™vesicle extruder (200-nm filter; Avestin, Ottawa, Canada) the membrane vesicles were aliquoted and stored at –80 °C.

Photoaffinity Labeling of Mrp1 Protein—Vanadate preparation and photoaffinity labeling of Mrp1 protein were performed according to procedures described previously (26). The amount of membrane vesicles, [32P]-labeled nucleotide, vanadate, NEM treatment, incubation time, and temperature are specifically indicated in Figs. 3–6. In order to determine which NBD was labeled, the samples after UV irradiation were digested with TPCK-treated trypsin (26). The amount of TPCK-treated trypsin is indicated in Figs. 3 and 4. The samples after trypsin digestion were diluted in 0.5 ml of radioimmunoprecipitation buffer containing 50 mm Tris-HCl (pH 7.4), 150 mm NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1× protease inhibitors, 5 µl of 42.4 antibody against NBD1 and 5 µl of 897.2 antibody against NBD2 (26) were added to the solution and gently shaken overnight in a cold room (4 °C). Then protein G-agarose beads were added to the mixture and incubated for 60 min in a cold room. The complex formed with the protein G-agarose beads were washed four times with radioimmunoprecipitation buffer, eluted with 1× electrophoresis sample buffer for 60 min at room temperature, and then electrophoresed.

Membrane Vesicle Transport—ATP-dependent transport of 3H-labeled leukotriene C4 (LTC4) into the membrane vesicles was assayed by a rapid filtration technique (37, 38). The assays were carried out in a 30-µl solution containing 3 µg of membrane vesicles, 50 µl Tris-HCl (pH 7.5), 250 mm sucrose, 10 mm MgCl2, 200 mm LTC4 (17.54 nCi of 3H-labeled LTC4), and varying concentrations of ATP as indicated in the figures. After incubation at 37 °C for 8 min, the samples were brought back to ice and diluted with 1 ml of ice-cold 1× transport buffer (50 mm Tris-HCl, pH 7.5, 250 mm sucrose, and 10 mm MgCl2) and trapped on nitricellulose membranes (0.2 µm) that had been equilibrated with 1× transport buffer. The filter was then washed with 10 ml of ice-cold 1× transport buffer, air-dried, and placed in a 10 ml of biodegradable counting scintillant (Amersham Biosciences). The radioactivity bound to the nitrocellulose membrane was determined by liquid scintillation counting (Beckman LS 6000SC).

RESULTS

Cysteine Substitutions in Mrp1 Walker A Motifs Do Not Compromise Protein Maturation—Fig. 5A shows an alignment of Walker A motifs from several ABC transporters including P-gp, Mrp1, and Mdr. The cysteine residue between the second and third glycines is conserved in the two Walker A motifs of the NBDs of P-gp. These two NBDs have been shown to be functionally equal (17–22, 30). Complete inactivation of P-gp ATPase activity by N-ethylmaleimide required the incorporation of 2 NEM molecules per P-gp molecule (39, 40). However, the concentrations of NEM required for half-maximal inactivation of ATPase activity were different for the two NBDs (41). At the corresponding position in Walker A motifs of Mrp1 there is a C in NBD1 and an A in NBD2. The role played by the
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Fig. 4. $K_m$ values of NBD1 and NBD2 determined in wild-type MRP1 protein. The photolabeling was carried out in a 10 μl of solution containing 10 μg of MRP1 membrane vesicles and varying concentrations of [α-32P]8-N3-ATP indicated. The samples were incubated on ice for 1 min, digested with TPCK-treated trypsin (trypsin/protein mass ratio was 1:32) for 15 min at 37 °C, and immunoprecipitated with antibodies against NBD1 (42.4) and NBD2 (89.2). The immunoprecipitated samples were subjected to SDS-PAGE and electroblotted to a nitrocellulose membrane. A, autoradiogram. Molecular weight markers are indicated on the left. MRP1 indicates the intact 190-kDa MRP1 protein and higher molecular weight aggregation products caused by immunoprecipitation and verified by Western blot using a monoclonal antibody against MRP1. NBD1 and NBD2 indicate [α-32P]8-N3-ATP incorporated into either NBD1 or NBD2 fragments. B, ratio of the labeled NBD1 versus NBD2 fragments. The amounts of [α-32P]8-N3-ATP incorporated into either NBD1 or NBD2 fragments were measured by electronic autoradiography (Packard Instant Imager), and the ratio of NBD1/NBD2 is from comparing the counts in NBD1 (119 ± 53 kDa) and NBD2 (65 ± 39 ± 31 kDa). The data are the average of three experiments with S.D. bars indicated. C, plot of the amount of [α-32P]8-N3-ATP incorporated into NBD1 fragments versus [α-32P]8-N3-ATP concentrations. The amounts of [α-32P]8-N3-ATP incorporated into NBD1 measured in A were plotted against [α-32P]8-N3-ATP concentrations and yielded a $K_m$ value of $14.3 ± 2.5 \mu M (n = 3)$. D, plot of the amount of [α-32P]8-N3-ATP incorporated into NBD2 fragments versus [α-32P]8-N3-ATP concentrations. The amounts of [α-32P]8-N3-ATP incorporated into NBD2 measured in A were plotted against [α-32P]8-N3-ATP concentrations and yielded a $K_m$ value of $29.5 ± 5.3 \mu M (n = 3)$. The double mutant C682A/A1331C was determined in Fig. 2A to reflect a different amount of MRP1 protein. The experiments in Fig. 2B were performed using the same amount of MRP1 protein in a total of 3 μg of membrane vesicle protein.

The corresponding residue (Ser12) in MJ0796 in ATP binding is to provide a backbone nitrogen to interact with the β-phosphate of the bound ATP (42). For that purpose, it should not be matter whether this position is occupied by cysteine, serine, or alanine. However, since the two NBDs of MRP1 protein have different residues at this position, this may contribute to their different properties with respect to ATP binding and hydrolysis. To test this possibility, we decided to interchange these two residues in the two NBDs (Fig. 1B). Fig. 1C shows that C682A, A1331C, and C682A/A1331C generated mature protein like wild-type MRP1, indicating that these mutations did not affect normal protein processing, although the amounts of MRP1 protein in the membrane vesicles were slightly different from each other (Fig. 1C).

The A1331C Mutation in NBD2 Increased the $K_m$ for ATP in MRP1-mediated LTC4 Transport—In order to test whether these Walker A mutations, C682A in NBD1 and A1331C in NBD2, affect the ATP-dependent LTC4 transport, membrane vesicles prepared from BHK cells expressing these variant MRP1s were utilized to determine the $K_m$ (ATP) and $V_{max}$ (LTC4) values. Fig. 2A and Table I show that the $K_m$ (ATP) of wild-type MRP1 is ~59 μM, which is similar to the previously reported $K_m$ value (38). The $K_m$ value for NBD1 mutant C682A decreased to 20 μM, whereas the $V_{max}$ of LTC4 transport increased slightly from 230 pmol/min (wild type) to 272. In contrast, the mutation in NBD2, A1331C, greatly increased the $K_m$ (ATP) to 255 μM ATP, whereas the $V_{max}$ of LTC4 transport was not changed (Table I), implying that this NBD2 mutation greatly affects the ATP binding/hydrolysis at NBD2. Interestingly, although the $K_m$ (ATP) of the NBD1 mutant C682A was decreased, the $K_m$ (ATP) of the double mutant C682A/A1331C was much higher than either the NBD1 mutant C682A or the wild type but similar to the NBD2 mutant A1331C, implying that ATP binding/hydrolysis at NBD2 played a dominant role in ATP-dependent LTC4 transport.

The $V_{max}$ is expressed in units of pmol of LTC4 transported/mg of protein/min, the amount of MRP1 protein in the membrane vesicles is an important factor determining the $V_{max}$ value. As indicated in Fig. 1C, the amounts of MRP1 proteins were different between wild-type and the mutants, and the above experiments were performed using the same amount of total membrane vesicle protein; therefore, the $V_{max}$ (LTC4) determined in Fig. 2A reflected a different amount of MRP1 proteins. The experiments in Fig. 2B were performed using the same amount of MRP1 protein in a total of 3 μg of membrane vesicle protein (adjusted by adding varying amounts of membrane vesicles prepared from parental BHK cells without MRP1). The order of the amount LTC4 transported is C682A > wild-type > A1331C > C682A/A1331C, which is consistent with the order of the $V_{max}$ values of wild-type and mutant MRP1s.

Cys to Ala Substitution in NBD1 (C682A) Increased the Affinity, whereas Ala to Cys Substitution in NBD2 (A1331C) Decreased the Affinity for ATP Binding—The $K_m$ (ATP) values in Table I implied that the mutation in NBD1, C682A, increased the affinity for ATP, whereas the mutation in NBD2, A1331C, decreased the affinity for ATP. However, the $K_m$ (ATP) value of the double mutant C682A/A1331C was similar to the NBD2 mutant A1331C. We postulated the following: 1) ATP binding to NBD1 of C682A/A1331C might be similar to NBD1 of C682A, whereas ATP binding to NBD2 of C682A/A1331C might be
FIG. 5. N-ethylmaleimide has a bigger effect on ATP labeling at cysteine residue in NBD1 than in NBD2. 10 μg of membrane vesicles were incubated in 20 μl of solution containing 10 mM Tris-HCl, pH 7.5, 250 mM sucrose, 0.2 mM MgCl₂, 5% ethanol, and varying concentrations of NEM indicated in the figure at 37 °C for 5 min. 20 mM cysteine was added to the reaction mixture and incubated at 37 °C for 6 min. The membrane proteins were then pelleted in a microcentrifuge in a cold room (4 °C). The pellet was resuspended into 10 μl of solution containing 10
similar to NBD2 of A1331C; 2) ATP binding and hydrolysis at NBD2 might play a dominant role in ATP-dependent LTC4 transport, leading to the similar K_{a/ATP} values of A1331C and C682A/A1331C. If that is the case, the ATP binding to C682A should be similar to C682A/A1331C. In order to test this possibility, membrane vesicles containing these proteins were labeled with [α-^{32}P]-8-N3ATP on ice for 1 min without vanadate. Under these conditions, vanadate-dependent trapping of ADP, the product of ATP hydrolysis, was completely abolished. Therefore, the labeling mainly reflects ATP binding to these proteins. Table II shows that the mutation in NBD1, C682A, decreased the K_{v} from 20.5 μM ATP (wild-type) to 8.5, implying increased affinity for ATP. The K_{v} value of A1331C was very similar to wild-type protein (17.0 versus 20.5), implying that the K_{v} value of A1331C was mainly attributable to the ATP binding to NBD1. Interestingly, although the K_{a/ATP} value of C682A/A1331C was ~10-fold higher than C682A (Table I), the K_{v} value of C682A/A1331C was almost identical to the value of C682A (Table II, 9.0 versus 8.5), further supporting our conclusion that the K_{v} value was mainly attributed to ATP binding to the C682A-mutated NBD2. The high K_{a/ATP} value of C682A/A1331C was interpreted as reflecting greatly reduced ATP binding at the mutated NBD2 (A1331C). To test this possibility, the membrane vesicles containing different MRP1 variants were labeled with [α-^{32}P]-8-N3ATP at either 0 or 37 °C and then digested with trypsin to separate NBD1 and NBD2 (Fig. 3). The ratio of ATP bound to NBD1 versus NBD2 at 37 °C was ~0.3 for wild type and C682A (Fig. 3), consistent with our previous results (26), whereas these ratios of labeling at 37 °C for A1331C and C682A/A1331C were ~2.0 and 1.5 (Fig. 3), respectively. Therefore, the labeling of the mutated NBD2 in A1331C and C682A/A1331C at 37 °C was greatly diminished, implying that the replacement of the alanine residue with cysteine in the Walker A motif in NBD2 greatly decreased the affinity for ATP binding at NBD2. The ratio of labeling at 0 °C was ~1.6 and 1.3 for wild-type and C682A (Fig. 3), implying that NBD1 may have higher affinity for ATP binding than NBD2. Although the ratio of labeling at 0 °C for A1331C and C682A/A1331C was not determined because of very weak labeling at the mutated NBD2, it clearly showed that the labeling of NBD1 was much higher than that of NBD2 (Fig. 3), also implying that the replacement of the alanine residue with cysteine in Walker A motif in NBD2 greatly decreased the affinity for ATP binding at the mutated NBD2.

**NBD1 Has Higher Affinity for ATP than NBD2**—The results in Table II and Fig. 3 and previous findings (25, 26) imply that NBD1 of MRP1 may have higher affinity for ATP binding than NBD2. To determine the affinities for ATP binding to NBD1 and NBD2, membrane vesicles containing wild-type MRP1 were labeled with [α-^{32}P]-8-N3ATP on ice for 1 min in the absence of vanadate and digested with trypsin to separate NBD1 and NBD2 fragments (Fig. 4A). Fig. 4, C and D, shows that the K_{v} values of NBD1 and NBD2 are ~14 and 30 μM ATP, respectively, indicating that NBD1 has higher affinity for ATP binding than NBD2. Fig. 4B showed that the ratio of bound ATP at NBD1 and NBD2 was ~3 at 0.5 μM [α-^{32}P]-8-N3ATP and gradually decreased at nucleotide higher concentrations. The ratio approaches 1 at high nucleotide concentrations.

**Photolabeling of MRP1 with a Cysteine Residue in the NBD1 Walker A Motif Is Much More Sensitive to NEM Modification than the Protein with a Cysteine at the Corresponding Position in NBD2**—As shown in Table II, the primary sequence or the structure of NBD1 plays a decisive role in determining the overall K_{a/ATP} values of the proteins. Thus, ATP labeling of the protein containing a cysteine residue in the Walker A motif of NBD1 should be much more sensitive to NEM modification than the protein without a cysteine residue in that region. To test this possibility, membrane vesicles containing the variant MRP1s were treated with varying concentrations of NEM and then labeled with [α-^{32}P]-8-N3ATP (Fig. 5). The labeling of wild type (Fig. 5, A and B), C682A (Fig. 5, C and D), and A1331C (Fig. 5, E and F) at low concentrations of NEM was enhanced.

The mechanism of this enhancement is not yet known. However, the labeling in all cases was inhibited at higher concentration of NEM, yielding IC_{50} values (Table III) of 5.9 (wild-type MRP1), 69.4 (C682A), 3.7 (A1331C), and 29.3 μM NEM (C682A/A1331C). It is clear that the IC_{50} values of the proteins with a cysteine residue in the Walker A motif of NBD1, such as wild-type and A1331C, are much lower than the proteins without a cysteine residue in that region, such as C682A and C682A/A1331C, indicating that ATP binding to the proteins with a cysteine residue in NBD1 is much more sensitive to NEM modification than the proteins without this cysteine residue. The IC_{50} value for A1331C (with cysteine residues in both NBDs) is lower than that for wild-type protein (with a cysteine residue in NBD1) and the value for C682A/A1331C (with a cysteine residue in NBD2) is lower than that for C682A (without a cysteine residue in both NBDs), indicating that the cysteine residue in NBD2 also plays a role in the NEM inhibition.

**NBD1 Walker A Motif Is Much More Sensitive to NEM Modification than the Protein with a Cysteine at the Corresponding Position in NBD2**—The results in Tables I and II indicate that ATP hydrolysis at NBD2 plays a dominant role in determining the overall K_{a/ATP} values of the proteins. Thus, ATP labeling of the protein containing a cysteine residue in the Walker A motif of NBD1 should be much more sensitive to NEM modification than the proteins without this cysteine residue. The IC_{50} value of A1331C (with cysteine residues in both NBDs) is lower than that for wild-type protein (with a cysteine residue in NBD1) and the value for C682A/A1331C (with a cysteine residue in NBD2) is lower than that for C682A (without a cysteine residue in both NBDs), indicating that the cysteine residue in NBD2 also plays a role in the NEM inhibition.

**ATP-dependent LTC4 Transport of the Protein Containing a Cysteine Residue in the Walker A Motif of NBD1 Is Much Less Sensitive to NEM Modification than the Protein Containing a Cysteine Residue in the Walker A Motif of NBD2**—The results in Tables I and II indicate that ATP hydrolysis at NBD2 plays a dominant role in ATP-dependent LTC4 transport. Based on this rationale, a protein containing a cysteine residue in the Walker A motif of NBD2 should be much more sensitive to NEM modification than a protein without a cysteine residue in that region. To test this possibility, the influence of NEM on LTC4 transport was assayed by treating the membrane vesicles containing variant MRP1s with increasing concentrations of NEM. ATP-dependent LTC4 transports were inhibited completely by 1 mM NEM in all cases (Fig. 6). However, the sensitivities of the variant MRP1s to NEM differed greatly (Table IV). Those proteins with a cysteine residue in Walker A motif of NBD2, such as A1331C and C682A/A1331C, are much more sensitive to NEM than the proteins without a cysteine residue in that region.
sensitive to NEM modification than the proteins without this residue in that region, such as wild-type and C682A, suggesting that ATP binding and hydrolysis at NBD2 plays a dominant role in ATP-dependent LTC4 transport. In addition, the IC50 value for wild-type MRP1 (with a cysteine residue in the Walker A motif of NBD1) is lower than that for C682A (without a cysteine residue in both NBDs), indicating that ATP binding at NBD1 also plays a certain role in ATP-dependent LTC4 transport.

**DISCUSSION**

The Walker A consensus sequence GXXGXSXXGKS(S/T) and the Walker B consensus sequence XXXD(E/D) from one NBD and the ABC signature sequence LSGGQ from the other are involved in ATP binding by ABC proteins (42–44). The crystal structure of a bacterial ABC transporter, MJ0796, indicated that some nonconserved residues (X) may also contribute to ATP binding (42) (e.g. the γ-oxygen of Ser40 of MJ0796 interacts with the ω-phosphate of the bound ATP, whereas the peptide backbone nitrogen of Ser42 interacts with the β-phosphate of the bound ATP) (42). Comparison of the amino acid residues at the position Ser42 of MJ0796 in 18 Walker A motifs showed that nine of them are serine, five are cysteine, and four are alanine residues (Fig. 1A). This raises the question of whether the side chains of these residues play a role in ATP binding. There is a cysteine residue at this position in NBD1 of MRP1 but an alanine residue in NBD2 (Fig. 1A). The cysteine residue in NBD1 was mutated to alanine (Fig. 1B, C682A), and the alanine residue was mutated to cysteine in NBD2 (Fig. 1B, A1331C). The C682A mutation decreased the Km value (Table I) and the Kd value (Table II), indicating that C682A mutation increases the affinity for ATP. The Km values of A1331C and C682A/A1331C are ~4-fold higher than that of wild-type (Table I), which are not consistent with their Kd values (Table II). The Kd value of A1331C is almost identical to that of wild-type (Table II), whereas the Kd value of C682A/A1331C is almost identical to that of C682A (Table II). These results were interpreted as indicating that the Kd value for overall binding to both NBD1 and NBD2 in a full-length MRP1 protein was mainly attributed to ATP binding at NBD1. The results in

**Table IV**

| Protein         | Amino acid at position 682 (NBD1) | Amino acid at position 1331 (NBD2) | IC50a µM-NEM |
|-----------------|-----------------------------------|-----------------------------------|--------------|
| Wild-type MRP1  | Cys                               | Ala                               | 299.58 ± 1.85 |
| C682A           | Ala                               | Ala                               | 490.91 ± 8.82 |
| A1331C          | Cys                               | Cys                               | 8.92 ± 0.33  |
| C682A/A1331C    | Ala                               | Cys                               | 5.99 ± 0.42  |

a The experiments in Fig. 6 were performed in triplicate. IC50 values (n = 2) were derived from Fig. 6. IC50 value is the NEM concentration inhibiting 50% of ATP-dependent LTC4 transport by the MRP1 protein.

**FIG. 6.** N-Ethylmaleimide has a bigger effect on ATP-dependent LTC4 transport at cysteine residue in NBD2 than in NBD1. 3 µg of membrane vesicles were incubated in a 15-µl solution containing 10 mM Tris-HCl, pH 7.5, 250 mM sucrose, 0.2 mM MgCl2, 5% ethanol, and varying concentrations of NEM indicated in the figure at 37 °C for 30 min. At the end of NEM treatment, the volume of the reaction mixture was adjusted to 30 µl containing 4 mM ATP, 200 nM LTC4, 10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 0.2 mM MgCl2 and then further incubated at 37 °C for 6 min. The remaining steps of the membrane vesicle transport experiments were performed according to the method described under "Experimental Procedures." The experiments were performed in triplicate. The amount of LTC4 transported by each membrane vesicle with 0 µM NEM treatment was considered as 100%.
We had found that the nonhydrolyzable ATP analogue [α-32P]βγ-ATP-PNP bound primarily to NBD1 of wild-type MRP1 in the absence of other nucleotides (31), implying that NBD1 might have higher affinity for nucleoside triphosphate than NBD2. In another report, co-expression of two MRP1 half-molecules revealed strong labeling of NBD1 and weak labeling of NBD2 with [α-32P]βγ-ATP on ice (25), also hinting that NBD1 might have higher affinity for ATP than NBD2. To directly test this hypothesis, membrane vesicles containing wild-type MRP1 protein were photolabeled with [α-32P]βγ-ATP on ice and digested with TPCK-treated trypsin after UV irradiation (Fig. 4), yielding $K_m$ values of 14 and 30 μM for NBD1 and NBD2, respectively. These results indicate that NBD1 has a moderately higher affinity for ATP than NBD2. The results in Fig. 4B showed that the ratio of bound ATP at NBD1 versus NBD2 was $-3$ at 0.5 μM ATP and decreased to 1.3 at 64 μM, approaching 1 at higher ATP concentration, implying that both NBDs should always be filled inside a cell with ATP in the millimolar range. ATP is most likely always saturating inside a cell, so both sites would always be filled. However, if the ATP concentration in the microenvironment surrounding an MRP1 molecule should fall drastically after hydrolysis events and diffusion of ATP back into this microenvironment is not sufficiently rapid, then there may be a temporary period of low ATP concentration. In that situation, ATP will preferentially bind to NBD1, this binding then causing a conformational change to enhance ATP binding (31) and hydrolysis at NBD2, which is coupled to solute transport. In this way, ATP binding to NBD1 at low nucleotide concentration, but not at high concentration, would play a regulatory role. This molecular design of the MRP1 protein would guarantee that the molecule works very efficiently over a large range of ATP concentrations, including ones that are very low.

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Table I were interpreted as indicating that the $K_m$ value of a full-length MRP1 protein was mainly attributed to ATP binding/hydrolysis at NBD2. This interpretation means that the mutation of A1331C should greatly decrease affinity for ATP at NBD2. Indeed, the labeling of the NBD2 fragments of either A1331C or C682A/A1331C was greatly reduced (Fig. 3). In addition, the results of NEM modification also support the above conclusions. The NEM concentration required to inhibit 50% of $\left[32P\right]8$-N3ATP labeling of proteins with a cysteine residue in NBD1, such as wild-type and A1331C, is much lower than that of the proteins without a cysteine in that location, such as C682A and C682A/A1331C (Table III), indicating that ATP binding to NBD1 plays a dominant role in determining the $K_m$ value of a full-length MRP1 protein. In contrast, the NEM concentration required to inhibit 50% of ATP-dependent LTC4 transport by the proteins without a cysteine residue in NBD2, such as wild-type and C682A, is much higher than that of the proteins with a cysteine in NBD2, such as A1331C and C682A/A1331C (Table IV), implying that ATP binding/hydrolysis at NBD2 plays a dominant role in ATP-dependent LTC4 transport.

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ATP Binding to the First Nucleotide Binding Domain of Multidrug Resistance-associated Protein Plays a Regulatory Role at Low Nucleotide Concentration, whereas ATP Hydrolysis at the Second Plays a Dominant Role in ATP-dependent Leukotriene C4 Transport

Runying Yang, Liying Cui, Yue-xian Hou, John R. Riordan and Xiu-bao Chang

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