ATP Binding, Not Hydrolysis, at the First Nucleotide-binding Domain of Multidrug-Resistance-associated Protein MRP1 Enhances ADP·Vi Trapping at the Second Domain*

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Multidrug resistance is a major obstacle to successful chemotherapeutic treatment of many types of cancers. Over-expression of P-glycoprotein (P-gp)1 and/or multidrug resistance-associated protein (MRP1) confers resistance to a broad range of anti-cancer drugs (1, 2). Both proteins transport anticancer drugs out of cells in an ATP-dependent manner by utilizing their membrane-spanning domains and two nucleotide binding domains (NBDs) (3–5), i.e. they couple ATP binding and hydrolysis to transport of solutes (6–15). However, it is unknown whether they share the same mechanism of this coupling. In the extensively studied P-gp, the two NBDs have been shown to be functionally equivalent with identical ATP hydrolysis steps occurring alternately at each NBD (16–20) and coupling one transport event with one ATP hydrolysis (21). Ambudkar’s group (22) reported that there are two independent ATP hydrolysis events in a single drug transport cycle, one ATP hydrolysis is associated with efflux of drug, whereas the other causes conformational resetting to the original state of the molecule (23). However, in their interpretation the ATP binding/hydrolysis sites of P-gp are recruited in a random manner during hydrolysis (23), meaning that the two NBD sites are functionally equivalent. In other reports, the two NBDs of P-gp were found to be essential for its function but not entirely symmetric (24, 25). Vigano et al. (25) proposed recently that ATP binding/hydrolysis at NBD1 is associated with efflux of drug, whereas the event at NBD2 is associated with the “reset” of the molecule. Therefore, how events at NBD1 and NBD2 of P-gp cooperate during drug transport is still not clear. Considerable evidence has accumulated indicating that the two NBDs of some other ATP-binding cassette (ABC) transporters, including the sulfonylurea receptor (SUR1) (26, 27), cystic fibrosis transmembrane-conductance regulator (CFTR) (28, 29), and MRP1 (30–33), have very distinctive properties. For example, in the case of MRP1, the following points clearly indicate that its two nonequivalent NBDs have different properties and functions. First, modifications of the consensus Walker motifs in the two NBDs do not inactivate the protein completely and have different effects on solute transport (30–32). Second, photoaffinity labeling experiments with 8-azido-ATP also revealed an asymmetry between NBD1 and NBD2, with NBD1 preferentially labeled by 8-Ν9[γ-32P]ATP (30, 31), whereas NBD2 trapped the nucleoside diphosphate hydrolysis product (30, 31, 33). Third, the ATP binding/hydrolysis sites of MRP1 seem not to be recruited in a random manner because photolabeling by the nonhydrolyzable 8-Ν9[γ-32P]AMP-PNP occurred predominately at NBD1 (34), and the NBD1 fragment was labeled predominantly with 8-Ν9[γ-32P]ATP on ice in the dual-expressed N- and C-halfes of MRP1 (30). Fourth, ADP trapping at NBD2 enhances ATP binding at NBD1 (31), and ATP binding at NBD1 allosterically enhances ADP trapping or AMP-PNP binding at NBD2 (34), implying that ATP binding at NBD1 or ADP trapping at NBD2 induces conformational change of the MRP1 molecule. It seems likely that the conformational changes of the MRP1 molecule caused by ATP bind-

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1 The abbreviations used are: P-gp, P-glycoprotein; MRP1, multidrug resistance-associated protein; NBD, nucleotide binding domain; 8-Ν9ADP, 8-azidoadenosine 5′-diphosphate; AMP-PNP, adenosine 5′-[(β,γ-methylene)diphosphate]; ATP·Vi, adenosine 5′-O-(thiotriphosphate); DDM, N-dodecyl-β-

β-maltoside; BHK, baby hamster kidney.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Sodium orthovanadate, EGTA, ATP, ATPγS, AMP-PNP, AMP-PCP, LiCl, ouabain, and sheep brain lipid were purchased from Sigma. Formic acid and polyethyleneimine-cellulose plates were from Fisher. His-Bind Resin was fromNovagen. N-Dodecyl-beta-maltoside (DDM) was from Calbiochem. 8-N3[32P]ADP was purchased from Affinity Labeling Technologies. [γ-32P]ATP·S was purchased from Amersham Biosciences. Dulbecco’s modified Eagle’s medium/F-12 cell culture media were from Invitrogen. Hexokinase was from Roche Molecular Biochemicals. The Stratagene UV Crosslinker 2400 model (wavelength 254 nm) was from Stratagene.

**Stable Cell Line Expressing Wild-type MRP1 in BHK Cells and Cell Culture**—Stable cell line expressing wild-type MRP1 in baby hamster kidney (BHK-21) cells was established previously (31, 35). These cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal bovine serum and 150 mM methotrexate (the original colonies expressing MRP1 were selected in 500 mM methotrexate and decreased to 150 μM methotrexate because the lesser amount of this drug did not affect the expression of MRP1 protein) at 37 °C in 5% CO2. Cells for membrane vesicle preparations were grown in roller bottles (Belco) in Dulbecco’s modified Eagle’s medium/F-12 containing 5% fetal bovine serum and 150 μM methotrexate at 37 °C.

**Membrane Vesicle Preparations**—MRP1-containing membrane vesicles were prepared according to the procedure described previously (31). Briefly, the cells grown in roller bottles were collected by centrifugation, resuspended in membrane vesicle preparation buffer containing 10 mM Tris-HCl, pH 7.5, 250 mM sucrose, 0.2 mM MgCl2, and 1× protease inhibitors (2 μg/ml aprotinin, 121 μg/ml benzamidase, 3.5 μg/ml E64, 1 μg/ml leupeptin, and 50 μg/ml Pefabloc) and equilibrated on ice for 20 min at 800 g for 15 min in a Parr N2 cavitation bomb. After pressure release, the cell homogenate was adjusted to 1 mM EDTA. The homogenate was centrifuged at 10,000 g for 15 min. The supernatant was overlaid on a 10 mM Tris-HCl, pH 7.5, and 250 mM sucrose and centrifuged at 100,000 g for 45 min. The pellet was resuspended in a solution containing 10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 1× protease inhibitors. After passage through a Liposofast™ vesicle extruder (200-nm filter, Avestin, Ottawa, Canada), the membrane vesicles were aliquoted and stored in −80 °C.

**Hexokinase Treatment of ATP Analogues**—To remove the trace amount of contaminating ATP in ATP analogues, 5 mM AMP-PNP, AMP-PCP, or ATP-γS solutions were treated with hexokinase as described previously (36). Briefly, each nucleotide at 5 mM in a 1-ml solution containing 20 mM Tris-HCl, pH 7.5, and 1 mM EDTA and centrifuged at 16,000 g for 15 min. Membranes at the interface were collected, diluted 5-fold with a solution containing 10 mM Tris-HCl, pH 7.5, and 250 mM sucrose, and then centrifuged at 100,000 g for 45 min. The pellet was resuspended in a solution containing 10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 1× protease inhibitors. After passage through a Liposofast™ vesicle extruder (200-nm filter, Avestin, Ottawa, Canada), the membrane vesicles were aliquoted and stored in −80 °C.

**Photoaffinity Labeling of MRP1 Protein**—Vanadate preparation and photoaffinity labeling of MRP1 protein were performed according to procedures previously described (36). Briefly, the photolabeled nucleotides were incubated with 10 μM MRP1 expressing cells for 20 min at 30 °C, washed three times with Tris-HCl buffer containing 10 mM MgCl2, and centrifuged at 5,000 g for 5 min. The cell homogenate was adjusted to 1 mM EDTA. The homogenate was centrifuged at 16,000 g for 15 min. The supernatant was overlaid on a 10 mM Tris-HCl, pH 7.5, and 250 mM sucrose and centrifuged at 100,000 g for 45 min. The pellet was resuspended in a solution containing 10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 0.2 mM MgCl2, and the cell pellet was kept in a −80 °C freezer overnight. The cell pellet was resuspended in a solution containing 10 mM Heps, pH 7.2, 1 mM EDTA, and 1× protease inhibitors, and then the cells were transferred to a Dounce homogenizer. After seven strokes in this homogenizer, the same volume of a solution containing 10 mM Heps, pH 7.2, 1 mM EDTA, and 500 mM sucrose was added to the homogenizer, and then another six strokes were performed. Nuclei were removed by centrifugation at 300 × g for 15 min at 4 °C. Membranes were collected at 33,000 g for 45 min at 4 °C. The membrane pellet was resuspended in a binding buffer containing 20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 20% glycerol, 25 mM imidazole, 1% DDM, 0.4% sheep brain lipid, and 0.05% β-mercaptoethanol and sonicated for a short time on ice. The insoluble material was removed by centrifugation at 10,000 × g for 15 min. The supernatant was applied onto a His-Bind Resin column that was bound to the binding buffer. The column was washed with 6 column volumes of modified binding buffer containing 0.1% DDM and 25 mM imidazole (first wash), 6 column volumes of modified binding buffer containing 0.1% DDM and 40 mM imidazole (second wash), and 6 column volumes of modified binding buffer containing 20 mM Tris-HCl, pH 7.4, 0.1% DDM and 40 mM imidazole (third wash). The bound protein was eluted with 2 column volumes of buffer containing 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 20% glycerol, 0.1% DDM, and 0.05% β-mercaptoethanol. The eluate was dialyzed against a solution containing 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 20% glycerol, 0.1% DDM, and 0.05% β-mercaptoethanol.

**[γ-32P]ATP·S Hydrolysis**—The experiments were performed in a 15-μl solution containing 40 mM Tris-HCl, pH 7.5, 2 mM ouabain, 0.1 mM EGTA, 10 mM MgCl2, 100 μM [γ-32P] ATP·S (3 μCi), and 0.5 μg of purified MRP1 protein. The same amount of protein-free sheep brain lipid (26 μg/ml solution) as in the 0.5 μg of purified MRP1 protein was used as a negative control. The reaction mixture was brought back to ice, and 10% aliquots were spotted immediately on a polyethyleneimine-cellulose plate. The samples were chromatographed with 0.5 mM LiCl and 1 mM formic acid as the solvent. The amounts of intact [γ-32P]ATP·S and [35S]Phosphate in each reaction mixture were determined by electronic autoradiography using a Packard Instant Imager (Packard Instrument Co.).

**RESULTS**

**ATP Enhancement of ADP Trapping in MRP1 Protein Is Greatly Diminished on Ice**—We found that photolabeling of MRP1 protein with 8-N3[32P]ADP was enhanced 4-fold mainly at NBD2 (34), suggesting that ATP binding or hydrolysis at NBD1 caused conformational change of the protein and increased affinity for ATP at NBD2. However, these experiments were performed at 37 °C and did not distinguish whether ATP binding or hydrolysis caused the conformational change of the protein. To distinguish these two possibilities, the same photolabeling experiments were performed on ice. Fig. 1, A and B, showed that the trapping of ADP to the protein was enhanced ∼30–40% in the presence of 5 to 20 μM ATP. The trapping was inhibited almost 70% in the presence of 640 μM ATP, simply because of competition between ATP binding and ADP trapping. These results were interpreted in the following two ways: 1) ATP hydrolysis at NBD1 may be required to induce the conformational change of the molecule to enhance ADP trapping at NBD2 and limited ATP hydrolysis on ice greatly diminishes the enhancement effect; 2) ATP binding alone can cause a conformational change that increases ADP trapping at NBD2 at higher temperature, such as 37 °C, and the smaller augmentation of trapping by ATP on ice may reflect a membrane structure and/or MRP1 protein that are in a “frozen state.” Therefore these results cannot be used to distinguish the two possibilities mentioned above.

**Hexokinase-treated AMP-PNP Does Not Enhance ADP Trapping by MRP1**—The nonhydrolyzable ATP analogue, AMP-PNP, enhanced ADP trapping by MRP1 protein (34), support-
ing the hypothesis that nucleotide binding alone can cause the conformational change of the protein. However, this could be misleading if there is a trace amount of hydrolyzable nucleotide contaminant. This can be removed efficiently by treating the solution with hexokinase (36). The results in Fig. 2 show that AMP-PNP, after hexokinase treatment, did not enhance ADP trapping by MRP1 protein, but instead it inhibited ADP trapping. Previous results clearly indicated that 8-N3-[α-32P]ADP incorporated into MRP1 protein in the absence of ATP was considered as 100%. The results are the average of three independent experiments.

**Experimental Procedures**

**Fig. 1.** ATP-dependent enhancement effect on 8-N3-[α-32P]ADP trapping cannot enhance ADP trapping by MRP1. The photolabeling experiments were performed according to the procedures described under “Experimental Procedures” in a 10-μl reaction mixture containing varying concentrations of ATP, indicated above the gel, on ice for 10 min. A, autoradiogram of ADP trapping on MRP1 protein in the presence of varying concentrations of ATP. The molecular weight markers are indicated on the left. The arrow indicates the 8-N3-[α-32P]ADP-labeled 190-kDa MRP1 protein. The 45-kDa protein labeled by 8-N3-[α-32P]ADP was also present in the membrane vesicles prepared from the parental BHK cells and was not recognized by several different antibodies against MRP1 protein. B, plot of the amount of 8-N3-[α-32P]ADP incorporated into MRP1 protein versus ATP concentrations. The amounts of 8-N3-[α-32P]ADP incorporated into MRP1 protein in panel A were determined by electronic autoradiography (Packard Instant Imager) and plotted out against ATP concentrations. The amount of 8-N3-[α-32P]ADP incorporated into MRP1 protein in the absence of ATP was considered as 100%. The results are the average of five independent experiments.

**Fig. 2.** Hexokinase-treated AMP-PNP does not enhance 8-N3-[α-32P]ADP trapping to MRP1 protein. The photolabeling experiments were performed according to the procedures described under “Experimental Procedures” in a 10-μl reaction mixture containing varying concentrations, indicated above the gel, of hexokinase-treated AMP-PNP at 37 °C for 10 min. Hexokinase treatment of AMP-PNP solution is described under “Experimental Procedures.” A, autoradiogram of ADP trapping to MRP1 protein in the presence of varying concentrations of AMP-PNP. The molecular weight markers are indicated on the left. The arrow indicates the 8-N3-[α-32P]ADP-labeled 190-kDa MRP1 protein. B, plot of the amount of 8-N3-[α-32P]ADP incorporated into MRP1 protein versus AMP-PNP concentrations. The amounts of 8-N3-[α-32P]ADP incorporated into MRP1 protein in panel A were determined by electronic autoradiography and plotted out against AMP-PNP concentrations. The amount of 8-N3-[α-32P]ADP incorporated into MRP1 protein in the absence of AMP-PNP was considered as 100%. The results are the average of five independent experiments.

**Conformational Change of MRP1 Caused by ATP Binding**

**Experimental Procedures**

AMP-PNP, after hexokinase treatment, did not enhance ADP trapping by MRP1 protein, but instead it inhibited ADP trapping. Previous results clearly indicated that 8-N3-[α-32P]AMP-PNP bound to NBD1 of MRP1 (34). Therefore it seems likely that nucleotide binding alone, at least in the case of AMP-PNP, cannot enhance ADP trapping by MRP1.

**The Nonhydrolyzable ATP Analogue, AMP-PCP, Enhances ADP Trapping—**If nucleotide binding alone cannot cause the conformational change leading to increased trapping, then the binding of other nonhydrolyzable ATP analogues, such as AMP-PCP, should also not enhance ADP trapping. To test this hypothesis, both hexokinase-treated and untreated AMP-PCP were utilized in the ADP trapping experiments. Fig. 3, A and B, shows that AMP-PCP, which was not treated with hexokinase, enhanced ADP trapping ~50%, whereas the hexokinase-treated AMP-PCP did so by ~20% (Fig. 3, C and D). The enhancing effect of the untreated AMP-PCP is slightly greater than that of AMP-PCP (Fig. 3) and slightly less than that of ATP (34). AMP-PNP may have an effect on ADP trapping similar to that of ATP under other conditions, such as at 0 °C. The experiments in Fig. 4E were performed on ice and showed that ATP-PNP stimulated ADP trapping by MRP1 protein ~20–30% (Fig. 4F). These results imply that ATP-PNP binding alone can cause the underlying conformational change.

**ATP-PNP Binding without Hydrolysis Can Enhance ADP Trapping—**Thus far, we have not ruled out the possibility that the poorly hydrolyzable ATP analogue, ATP-PNP, might be hydrolyzed to some extent during the 10-min incubation period at 37 °C. Therefore we estimated how long it would take for one MRP1 molecule to hydrolyze one ATP-PNP molecule. Table I shows that it took ~20 min for one MRP1 molecule at 37 °C to hydrolyze one ATP-PNP. Therefore, ~50% of the ATP-PNP bound to MRP1 protein should be hydrolyzed during the 10-min of incubation at 37 °C. Hence, some hydrolysis did occur.

Because it takes ~20 min for one MRP1 molecule to hydrolyze one ATP-PNP, at shorter times hydrolysis is essentially negligible. The experiments in Fig. 5A were performed as follows.
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The pelleted membrane proteins containing MRP1 were resuspended on ice with a 10-μl ice-cold reaction mixture, transferred to a 37 °C water bath, and incubated for 1, 2, 4, 8, 16, or 32 min. The samples were brought back to ice after incubation for the indicated times at 37 °C and diluted with 500 μl of ice-cold Tris-EGTA buffer immediately, and then the membrane was pelleted by centrifugation in a cold room (4 °C). Therefore, the temperature inside of the tubes incubated at 37 °C for only 1 min should not be 37 °C at the beginning of the incubation, and the incubation time at 37 °C must be less than 1 min. The amount of ATP-Pi hydrolyzed during this short period must be much less than during the 32-min period (Fig. 5A, lane 32'). Yet, this sample (1-min incubation at 37 °C) had the greatest enhancing effect on ADP trapping (Fig. 5, A and C), indicating that ADP trapping at NBD1 was not responsible for the conformation change and that ATP-Pi binding alone was sufficient. Fig. 5C shows that the enhancing effects gradually decreased with incubation time. The mechanism of this diminution is not yet clear. However, one of the possible reasons is that the unstimulated ADP trapping in the absence of ATP-Pi gradually increased with incubation time (Fig. 5, A and B), implying that nucleotide binding at NBD1 requires ATP hydrolysis at NBD2 (34), implying that nucleotide binding alone can induce the conformational change. However, why was the enhancing effect of nucleotide binding at NBD2 required for induction of the conformational change? We have found that the binding of ATP at NBD1 enhances ADP trapping or AMP-PNP binding to NBD2, implying that ATP binding at NBD1 causes conformational change of the MRP1 molecule (34) and the trapping of ATP-Pi, mimicking the ATP hydrolysis intermediate ADP-Pi, enhances intact ATP binding at NBD1 (31), implying that ATP hydrolysis at NBD2 causes conformational change in the MRP1 molecule. These conformational changes may contribute to active solute transport by the molecule. However, it is not clear whether the enhancing effect of nucleotide binding at NBD2 requires ATP hydrolysis at NBD1. The nonhydrolyzable ATP analogue, AMP-PNP, slightly enhances ADP trapping to MRP1 protein (34), implying that nucleotide binding alone is sufficient to induce the conformational change. However, why was the enhancing effect of AMP-PNP much lower than that of ATP (34)? Interestingly, a poorly hydrolyzable ATP analogue, ATPγS, induced the conformational change to the same extent as ATP as determined by fluorescence quenching (38), implying that nucleotide binding alone may be responsible for induction of the conformational change. However, because ATPγS is a poorly hydrolyzable ATP analogue, the nucleotide used in the experiments might be hydrolyzed by MRP1. Therefore, the question of whether ATP binding or hydrolysis is required to induce the conformational change was still unanswered. Our present results clearly indicate that ATP binding alone is sufficient to induce the conformational change of MRP1.

DISCUSSION

Over-expression of MRP1 protein confers resistance to a broad range of anti-cancer drugs (2). Solutes (for example, anti-cancer drugs) are extruded out of cells by MRP1 protein in an ATP-dependent manner (7, 14, 37) by utilizing its two NBDs to bind and hydrolyze ATP. Both NBDs of MRP1 protein can bind nucleotides (30, 31, 33). However, the properties and the functions of the two NBDs do not seem to be equal as discussed in the Introduction. How are the events at NBD1 and NBD2 related during substrate transport? We have found that the binding of ATP at NBD1 enhances ADP trapping or AMP-PNP binding to NBD2, implying that ATP binding at NBD1 causes conformational change of the MRP1 molecule (34) and the trapping of ADP-Pi, mimicking the ATP hydrolysis intermediate ADP-Pi, enhances intact ATP binding at NBD1 (31), implying that ATP hydrolysis at NBD2 causes conformational change in the MRP1 molecule. These conformational changes may contribute to active solute transport by the molecule. However, it is not clear whether the enhancing effect of nucleotide binding at NBD2 requires ATP hydrolysis at NBD1. The nonhydrolyzable ATP analogue, AMP-PNP, slightly enhances ADP trapping to MRP1 protein (34), implying that nucleotide binding alone can induce the conformational change. However, why was the enhancing effect of AMP-PNP much lower than that of ATP (34)? Interestingly, a poorly hydrolyzable ATP analogue, ATPγS, induced the conformational change to the same extent as ATP as determined by fluorescence quenching (38), implying that nucleotide binding alone may be responsible for induction of the conformational change. However, because ATPγS is a poorly hydrolyzable ATP analogue, the nucleotide used in the experiments might be hydrolyzed by MRP1. Therefore, the question of whether ATP binding or hydrolysis is required to induce the conformational change was still unanswered. Our present results clearly indicate that ATP binding alone is sufficient to induce the conformational change of MRP1.

Fig. 3. AMP-PCP slightly enhances 8-N3[32P]ADP trapping to MRP1 protein. The photolabeling experiments were performed according to the procedures described under “Experimental Procedures” in a 10-μl reaction mixture containing varying concentrations, indicated above the gel, of either untreated (A) or hexokinase-treated (C) AMP-PCP at 37 °C for 10 min. Hexokinase treatment of AMP-PCP solution is described under “Experimental Procedures.” A, autoradiogram of ADP trapping to MRP1 protein in the presence of varying concentrations of AMP-PCP (untreated). The molecular weight markers are indicated on the left. The arrow indicates the 8-N3[32P]ADP-labeled 190-kDa MRP1 protein. B, plot of the amount of 8-N3[32P]ADP incorporated into MRP1 protein versus AMP-PCP concentrations. The amounts of 8-N3[32P]ADP incorporated into MRP1 protein in panel A were determined by electronic autoradiography and plotted out against AMP-PCP concentrations. The amount of 8-N3[32P]ADP incorporated into MRP1 protein in the absence of AMP-PCP was considered as 100%. The results are the average of three independent experiments. C, autoradiogram of ADP trapping on MRP1 protein in the presence of varying concentrations of hexokinase-treated AMP-PCP. D, plot of the amount of 8-N3[32P]ADP incorporated into MRP1 protein versus AMP-PCP concentrations. The amounts of 8-N3[32P]ADP incorporated into MRP1 protein in panel C were determined by electronic autoradiography and plotted out against hexokinase-treated AMP-PCP concentrations. The amount of 8-N3[32P]ADP incorporated into MRP1 protein in the absence of AMP-PCP was considered as 100%. The results are the average of five independent experiments.
Conformational Change of MRP1 Caused by ATP Binding

FIG. 4. ATP\(\gamma\)S enhances 8-N\(3\)\(\gamma\)32P\]ADP trapping to MRP1 protein. The photolabeling experiments were performed according to the procedures described under "Experimental Procedures" in a 10-µl reaction mixture containing varying concentrations, indicated above the gel, of either untreated (A) or hexokinase-treated (C) ATP\(\gamma\)S at 37 °C for 10 min. Hexokinase treatment of ATP\(\gamma\)S solution is described under "Experimental Procedures." A, autoradiogram of ADP trapping on MRP1 protein in the presence of varying concentrations of ATP\(\gamma\)S (not hexokinase-treated). The ATP\(\gamma\)S concentration in each reaction mixture is indicated above the gel. The molecular weight markers are indicated on the left. The arrow indicates the position of 8-N\(3\)\(\gamma\)32P\]ADP-labeled 190-kDa MRP1 protein. B, plot of the amount of ATP\(\gamma\)S incorporated into MRP1 protein versus ATP\(\gamma\)S concentrations. The amounts of ATP\(\gamma\)S incorporated into MRP1 protein in panel A were determined by electronic autoradiography and plotted out against ATP\(\gamma\)S concentrations. The amount of ATP\(\gamma\)S incorporated into MRP1 protein in the absence of ATP\(\gamma\)S was considered as 100%. The results are the average of two independent experiments. C, autoradiogram of ADP trapping to MRP1 protein in the presence of varying concentrations of hexokinase-treated ATP\(\gamma\)S. The ATP\(\gamma\)S (hexokinase-treated) concentration in each reaction mixture is indicated above the gel. D, plot of the amount of ATP\(\gamma\)S incorporated into MRP1 protein versus ATP\(\gamma\)S concentrations. The amounts of ATP\(\gamma\)S incorporated into MRP1 protein in panel C were determined and plotted out against hexokinase-treated ATP\(\gamma\)S concentrations. The results are the average of four independent experiments. E, autoradiogram of ADP trapping on MRP1 protein in the presence of varying concentrations of hexokinase-treated ATP\(\gamma\)S at 0 °C for 10 min. F, plot of the amount of ATP\(\gamma\)S incorporated into MRP1 protein versus ATP\(\gamma\)S concentrations. The amounts of ATP\(\gamma\)S incorporated into MRP1 protein in panel E were determined and plotted out against hexokinase-treated ATP\(\gamma\)S concentrations. The results are the average of four independent experiments. Because the enhancement effects are always calculated by comparing the amount of ATP\(\gamma\)S incorporated into MRP1 protein in the absence of ATP\(\gamma\)S, the amounts of ATP\(\gamma\)S incorporated into MRP1 protein at 0 and 37 °C are important factors. On the basis of 19 different experiments performed at 0 and 37 °C at the same time, the labeling at 37 °C in the absence of other nucleotides is 1.96 ± 0.67-fold higher than at 0 °C. The value in the control lane of C was 4044, whereas it was 2060 in the control lane of E (4044/2060 = 1.96).

### TABLE I

| Temperature | Time Sample | Gross amount of ATP\(\gamma\)S hydrolyzed* | ATP\(\gamma\)S hydrolyzed by MRP1 | ATP\(\gamma\)S hydrolyzed by MRP1 S (hexokinase-treated) | Mole of ATP\(\gamma\)S hydrolyzed* Mole of MRP1 protein | Time required to hydrolyze one ATP\(\gamma\)S by one MRP1\(\gamma\)² |
|-------------|-------------|------------------------------------------|---------------------------------|-----------------------------------------------|------------------------------------------------------|-----------------------------------------------|
| 0 °C        | 120 26 SBL  | 2.38 ± 0.27                             | 0.30 ± 0.02                     | 4.50 ± 0.3                                     | 1.71 ± 0.11                                         | 70.18 ± 4.51                                  |
| 37 °C       | 120 26 SBL | 2.62 ± 0.39                             | 0.25 ± 0.02                     | 3.87 ± 0.3                                     | 1.23 ± 0.05                                         | 20.98 ± 1.61                                  |
| 37 °C       | 30 0.5 MRP1| 2.63 ± 0.25                             | 0.40 ± 0.07                     | 6.00 ± 1.05                                    | 2.28 ± 0.40                                         | 26.33 ± 4.62                                  |
| 37 °C       | 60 0.5 MRP1| 2.90 ± 0.36                             | 1.02 ± 0.12                     | 15.30 ± 2.1                                    | 5.82 ± 0.80                                         | 20.02 ± 2.53                                  |

* 0.5 µg of purified MRP1 protein was in 26 µg of sheep brain lipid (SBL). Therefore 26 µg of sheep brain lipid was used as a negative control.

² The experiments were performed in triplicates, and individual experiments were repeated four times. The results are the average of these four experiments.

* The percentage of ATP\(\gamma\)S hydrolyzed by MRP1 protein was calculated by subtraction of the percentage of ATP\(\gamma\)S hydrolyzed by 26 µg of sheep brain lipid from the 0.5 µg of purified MRP1 under the same condition.

* Picomoles of ATP\(\gamma\)S hydrolyzed was calculated by the percentage of ATP\(\gamma\)S hydrolyzed by the 0.5 µg of purified MRP1 protein times the total amount of ATP\(\gamma\)S in this 15-µl reaction mixture.

* 0.5 µg of purified MRP1 protein is considered as 2.63 pmol of MRP1 protein. Mole of ATP\(\gamma\)S hydrolyzed/mole of MRP1 protein is calculated by dividing pmol of ATP\(\gamma\)S hydrolyzed with 2.63 pmol of MRP1 protein.

* Time (min) required to hydrolyze one ATP\(\gamma\)S by one MRP1 molecule is calculated by dividing the incubation time with mole of ATP\(\gamma\)S hydrolyzed/mole of MRP1 protein.

protein. The results in Fig. 2 show that hexokinase-treated AMP-PNP cannot enhance ADP trapping, implying that AMP-PNP binding to NBD1 (34) cannot induce the necessary conformational change. If nucleotide binding alone cannot induce the conformational change, then the binding of other nonhydrolyzable ATP analogues should also not induce the conformational
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Fig. 5. ATPγS hydrolysis is not required for enhancing 8-N[α-32P]ADP trapping to MRP1 protein. The photolabeling experiments were performed according to the procedures described under "Experimental Procedures" in a 10-μl reaction mixture containing either no ATPγS (− lanes) or 20 μM hexokinase-treated ATPγS (+ lanes). The samples were resuspended in a 10-μl ice-cold reaction mixture on ice, transferred immediately to a 37 °C water bath, and then incubated at 37 °C for the indicated time. Lanes 0, − and +, the samples were incubated on ice for 1 min without transferring to 37 °C water bath. The other samples were incubated at 37 °C for 1 min (1′, − and +), 2 min (2′, − and +), 4 min (4′, − and +), 8 min (8′, − and +), 16 min (16′, − and +), and 32 min (32′, − and +). The samples were washed with 500 μl of ice-cold Tris-EGTA buffer immediately after the 37 °C incubation and UV irradiated on ice for 2 min. Because the samples were transferred from 0 to 37 °C directly, the temperature inside of the tubes was not 37 °C at the beginning of the 37 °C incubation. A, autoradiogram of ADP trapping to MRP1 protein in the absence or presence of 20 μM hexokinase-treated ATPγS. The − or + signs above the gel indicate the condition of the absence or presence of 20 μM of hexokinase-treated ATPγS in each of the specific reaction. The molecular weight markers are indicated on the left. The arrow indicates the 8-N[α-32P]ADP-labeled 190-kDa MRP1 protein. B, plot of the amount of 8-N[α-32P]ADP incorporated into MRP1 protein. The amounts of 8-N[α-32P]ADP incorporated into MRP1 protein in panel A were determined by electronic autoradiography. C, enhancement effect of ATPγS on ADP trapping. The amount of 8-N[α-32P]ADP incorporated into MRP1 protein in the absence of ATPγS was considered as 100%. The results are the average of four independent experiments.

change. However, in contrast to AMP-PNP, another nonhydrolyzable ATP analogue, AMP-PCP, can enhance ADP trapping (Fig. 3), implying that nucleotide binding alone can induce the conformational change of the protein. Interestingly, the enhancing effect of AMP-PCP on ADP trapping was much less than that of ATP, perhaps because of the structural difference between AMP-PCP and ATP. If the structure difference between nucleotides is a major factor determining the enhancing effects, then a nucleotide with a structure similar to that of ATP, such as ATPγS, should have a similar effect. Indeed, the hexokinase-treated ATPγS had an effect similar to that of ATP (Fig. 4), although we had not ruled out the possibility that ATPγS might be hydrolyzed during the 10-min incubation at 37 °C. The results shown in Table I and Fig. 5 clearly indicate that ATPγS binding alone, not hydrolysis, can enhance ADP trapping in MRP1 protein. However, nucleotide binding on ice (30) greatly diminished the stimulatory effect (Figs. 1 and 4), implying that ATP binding alone can induce the conformational change only under proper conditions such as those of temperature. In conclusion: 1) nucleotide hydrolysis at NBD1 is not required to induce the conformational change stimulating ADP trapping at NBD2; 2) nucleotide binding alone under proper conditions is sufficient to induce the conformational change; 3) the proper steric structure of the γ-phosphate of the nucleotide is a crucial factor affecting the ability of the nucleotide to induce the conformational change; when nitrogen replaces oxygen between the β and γ phosphates in AMP-PNP, this eliminates the enhancing effect, whereas when carbon replaces oxygen in AMP-PCP this greatly reduces the enhancing effect; 4) the replacement of oxygen with sulfur on the γ-phosphate in ATPγS also slightly reduces the enhancing effect.

Fig. 5 continued...

How does ATP binding to NBD1 induce the conformational change? The original "unexcited" structure of MRP1 protein should be the same no matter whether ATP, ATPγS, AMP-PNP, or AMP-PCP has been utilized to excite MRP1 protein. The only difference between ATP, AMP-PNP, and AMP-PCP is the atom between the β and γ-phosphates, which determines the distance and angle between the β and γ-phosphates (39). Therefore the steric structure of the γ-phosphate is the crucial determinant of the structural perturbation. Which residues of MRP1 protein interact with the γ-phosphate when the nucleotide binds to NBD1? Although the three-dimensional structure of MRP1 protein has not been solved, the structures of other ATP-binding cassette transporters, such as periplasmic histidine permease of Salmonella typhimurium (40) and MJ0796 from Methanococcus jannaschii (41), may provide a clue. By analogy with these, Ser-685 (a residue in Walker A of NBD1), Gln-713 (γ-phosphate linker), and Asp-792 (a residue in Walker B of NBD1) may work with the γ-phosphate via Mg2+. Lys-684 (a residue in Walker A of NBD1) may interact with the γ-phosphate directly. Asp-793 may function as a catalytic base to attack the water molecule to hydrolyze the bound ATP, Val-680 and Gly-681 (residues in the Walker A motif of NBD1) and Ser-1431, Val-1432, and Gly-1433 (residues in the ATP-binding cassette signature sequence of NBD2) may interact with the γ-phosphate. Upon ATP binding to NBD1 the γ-phosphate of the bound nucleotide either "pushes" or "pulls" (by electrostatic interactions between the γ-phosphate and the charged residues) some of these residues, leading to the conformational change and increasing the affinity for ATP at NBD2 (34). Changing of the atom between the β and γ-phosphates changes the spatial orientation of the γ-phosphate, either eliminating (such as AMP-PNP) or diminishing (such as AMP-PCP) the pushing or pulling force. If that is the case, mutations of these residues may affect ATP binding at NBD1 and decrease the ATP enhancing effect on ADP trapping. Indeed, mutations of K684L and D792A greatly diminish the ATP enhancing effect on ADP trapping (34). Interestingly, mutation of D792E, which did not significantly change the negative charge at that position, also greatly diminished the ATP enhancement effect on ADP trapping, indicating that the distance between the negative charged residue, Asp-792, and the γ-phosphate of ATP is also very important.

2 Y.-x. Hou, J. R. Riordan, and X.-b. Chang, unpublished results.
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Although these data provide evidence that ATP binding, not hydrolysis, at NBD1 is sufficient to induce the conformational change and enhance nucleotide binding at NBD2, they do not speak directly to how the protein couples ATP hydrolysis to solute transport. Upon binding of ATP to NBD2 there should be a transient state in which both NBDs bound ATP. Whether the bindings of ATP to both NBD1 and NBD2 will lead to the formation of a transient ATP sandwich (41–43) between NBD1 and NBD2 is not known. However, the efficient hydrolysis of the bound ATP at NBD2 (30, 31) may cause conformational change and enhance nucleotide binding at NBD2, they do not hydrolysis, at NBD1 is sufficient to induce the conformational change and enhance nucleotide binding at NBD2. If that is the case, whether the ATP bound at NBD1 is released during the incubation period at 37°C. If it is the case, whether the ATP bound at NBD1 is released as an intact ATP or hydrolyzed first and then released is not known. No matter how the ATP bound at NBD1 is released, the releasing of this bound nucleotide may bring the MRP1 molecule back to the original unexcited state so that the MRP1 molecule can start another cycle of solute transport.

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