

# The relationship between MRP1 activities and its NBD conformational changes

HUANG Zhenhua & HUANG Youguo

National Laboratory of Biomacromolecules, Center for Structural and Molecular Biology, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

Correspondence should be addressed to Huang Youguo (email: huang@sun5.ibp.ac.cn)

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**Abstract** MIANS, a sulfhydryl-reactive fluorescence, was used to label the cysteines of MRP1 (multidrug resistance protein), and the results indicated that an increase in fluorescence intensity and a large emission blue shift took place after two Cys residues of MRP1 reacted with MIANS, which demonstrated that labeled Cys residues in MRP1 reside in a relatively hydrophobic environment. The experimental results obtained from fluorescence resonance energy transfer further uncover that two Cys residues of MRP1 modified by MIANS located in the vicinity of its NBDs, of which one lies close to NBD1, and the other near NBD2. ATP, ADP and anticancer drugs can all reduce the rate of reaction of MRP1 with MIANS. The collisional quenchers, acrylamide, I<sup>-</sup>, and Cs<sup>+</sup> were used to assess local environments of MIANS bound to MRP1 and the results showed that the region around the MIANS-labeled cysteine is positively charged. Both MIANS and NEM, which are sulfhydryl-reactive reagents, inhibited MRP1 ATPase activity, whereas anticancer drugs activated it. These results demonstrated that all nucleotides and drugs could induce changes in conformation of the NBDs in MRP1. Nucleotides can bind directly to NBDs, but drugs may react first with TMDs, which in turn alters the accessibility of the two Cys residues bound by MIANS and affects MRP1 ATPase activity, which is coupled with the transport of its substrates. Taken together, the above experimental results provide direct evidence for further study on the coupling of translocation of the transported species to hydrolysis of ATP in MRP1.

**Keywords:** MRP1, multidrug resistance, conformation, coupling.

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The term multidrug resistance (MDR) is classically used to define a resistance phenotype where cells become resistant simultaneously to different drugs with no obvious structural resemblance and with different cellular targets. For some time after P-gp, the first membrane protein related with MDR, was discovered in 1976<sup>[1]</sup>, it was widely believed that P-gp was the exclusive cause of multidrug resistance. However, increasing evidence afterward suggested that this was not the case. Several cell lines were iso-

lated and displayed a multidrug resistance phenotype in the absence of detectable P-glycoprotein expression<sup>[2,3]</sup>. In 1992, Cole et al. reported another membrane protein, MRP1, which is also related to MDR<sup>[4]</sup>. Both P-gp and MRP1 are members of the ATP-binding cassette (ABC) transporter superfamily containing two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs), but MRP1 contains an additional TMD, which includes five transmembrane segments and an extracytosolic NH<sub>2</sub>-terminus<sup>[5-7]</sup>. It is

believed that two nucleotide binding domains (NBD1 and NBD2) of P-glycoprotein appear to be functionally equivalent, and a strongly supported model proposes that ATP hydrolysis occurs alternately at each NBD<sup>[8]</sup>. But it is not the cases for MRP1. Its two NBDs are much less similar than for P-glycoprotein, so the structure and function of the two NBDs are different. It had been reported that ATP binds exclusively at NBD1 and ADP traps predominantly at NBD2<sup>[9]</sup>.

As most of ABC transporters, the TMDs of MRP1 are important determinants of the different substrate specificities, and its NBDs are believed to bind and subsequently hydrolyze ATP to provide the energy for substrate transport<sup>[10]</sup>. Because it is not easy to obtain enough purified MRP1, there are not only few reports involved in the interaction of TMDs with NBDs of MRP1 but also few reports engaged in the mechanism on the coupling of translocation of the transported species to hydrolysis of ATP<sup>[11]</sup> in the protein. To illuminate the drug-resistant mechanism of MRP1 at molecular level, we purified MRP1 and tried to provide direct evidence to uncover the relationship between ATPase activity and the conformation change of TMDs and NBDs of MRP1 and its substrate transport.

## 1 Materials and methods

### 1.1 Materials

3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), asolectin, N-ethylmaleimide (NEM), dithioerythritol (DTE), Na<sub>2</sub>-ATP, imidazole, vincristine, vinblastine, daunorubicin, doxorubicin were purchased from Sigma Chemical Co. 2-(4'-maleimidylanilino) naphthalene- 6-sulfonic acid, sodium salt (MIANS), 2'-(or-3')-O-(trinitrophenyl) adenosine 5'-triphosphate, trisodium salt (TNP-ATP), 2'-(or-3')-O-(trinitrophenyl) adenosine 5'-diphosphate, disodium salt (TNP-ADP) were from Molecular Probes. MRPr1, a monoclonal antibody of MRP1, was purchased from ALEXLS Biochemicals. The other reagents were of analytical grade.

### 1.2 Purification of MRP1 protein

A baby hamster kidney cell line stably expressing

human MRP1/His<sub>10</sub>, a generous gift from Johnson Medical Research Center, Arizona, USA, was utilized for purification of MRP1 protein<sup>[12]</sup>. Cells expressing MRP1/His<sub>10</sub> were grown as adherent monolayer in DMEM (50%) and F-12 (50%) medium (pH 7.45) at 37°C in 5% CO<sub>2</sub> supplemented with 5% heat-inactivated fetal calf serum, 100 U/mL penicillin and 100 U/mL streptomycin and in the presence of 100 μmol/L methotrexate (to prevent cDNA of MRP1 from losing). Exponentially growing cells were used in all experiments.

MRP1 was purified as described previously<sup>[12]</sup> with a modification to use CHAPS as a substitute for *n*-dodecyl-β-D-maltoside (DDM) to solubilize cell membrane. Exponentially growing cells were solubilized in a buffer A (20 mmol/L Tris-HCl, pH 7.9, and 500 mmol/L NaCl) containing 5 mmol/L imidazole, 10 mmol/L CHAPS and 20% (v/v) glycerol. The solubilized sample was sonicated and insoluble material was removed by centrifugation at 10000 *g* for 15 min<sup>[12]</sup>. The supernatant was applied onto a His.Bind Resin column, which was pre-equilibrated in a buffer A, followed by three washes with 10 column volumes of buffers: first wash with buffer A containing 5 mmol/L CHAPS and 25 mmol/L imidazole, second wash with buffer A containing 5 mmol/L CHAPS, 10% glycerol, and 40 mmol/L imidazole and third wash with buffer B containing 20 mmol/L Tris-HCl (pH 7.4), 500 mmol/L NaCl, 5 mmol/L CHAPS and 40 mmol/L imidazole. The protein was eluted with 2 column volumes of buffer C containing 20 mmol/L Tris-HCl (pH 7.4), 5 mmol/L CHAPS, 0.05% β-mercaptoethanol and 300 mmol/L imidazole. The eluate was dialyzed extensively against 50 volumes of the buffer containing 5 mmol/L Tris-HCl (pH 7.4), 5 mmol/L CHAPS, 500 mmol/L NH<sub>4</sub>Cl and 0.05% β-mercaptoethanol in a cold room. The purified MRP1 protein was stored at -80°C after being ultrafiltrated.

### 1.3 Assay of MRP1 ATPase activity

The ATPase activity of MRP1 was determined as described previously<sup>[13]</sup> by using a colorimetric method to measure the release of inorganic phosphate from ATP. Typically, 1 μg of the purified MRP1 pro-

tein was incubated in buffer D (50 mmol/L Tris-HCl, pH 7.4, 2 mmol/L ATP, 5 mmol/L MgCl<sub>2</sub>, 0.5 mg/mL aroclorin) at 37°C in a 96-well microtitre plate (final volume 90 μL). To initiate the reaction, 10 μL of ATP solution (final concentration 2 mmol/L) in buffer D was added. After incubating for 4 h at 37°C, the reaction was stopped by the addition of 100 μL of 6% SDS/3% ascorbate/0.5% ammonium molybdate in 0.5 mol/L HCl. Products were stabilized by the addition of 100 μL of 2% sodium citrate/2% acetic acid. The absorbance at 750 nm in each well was measured using a microplate reader (Bio-RAD model 3550). Background absorbances were determined by performing the assay with heat-inactivated samples and were subtracted from the experimental sample. Various concentrations of sulfhydryl-reactive agents and anticancer drugs were added into the reaction system and the absorbances were measured to detect their effect on MRP1 ATPase activities.

#### 1.4 Fluorescence changes of MRP1 labeled by MIANS

MIANS was dissolved at 2.5 mmol/L in methanol, and the actual concentration was determined using absorbance measurements ( $\lambda_{\text{max}} = 322 \text{ nm}$ ,  $\epsilon = 17000 \text{ mol}^{-1} \text{ cm}^{-1}$ )<sup>[14]</sup>. Fluorescence was measured at 22°C using a Hitachi F-4010 fluorescence spectrophotometer (Ex = 322 nm, Em = 420 nm and slits = 5 nm). To determine the rate of MIANS reaction with Cys residues in MRP1, the protein (50 μg MRP1/mL in 500 μL buffer D) was preincubated with buffer D or increasing concentration of ATP, ADP and drugs at 22°C for 5 min. Labeling was initiated by addition of 4 μL of 0.5 mmol/L MIANS to 500 μL of MRP1 solution to give a final concentration of 4 μmol/L, and fluorescence was monitored continuously at an emission wavelength of 420 nm.

#### 1.5 Stoichiometry of MIANS labeling with MRP1

To determine the stoichiometry of labeling, 190 μg of MRP1 (1 mg/mL) in buffer D was treated with increasing concentration of MIANS for 20 min at 22°C in the dark. Unreacted MIANS was quenched with DTE, and the samples were dialyzed against 3 × 1 L of

50 mmol/L ammonium bicarbonate at 4°C in the dark. The MRP1 samples were then lyophilized and redissolved in 6 mol/L guanidine hydrochloride at a concentration of 200 μg/mL. Covalently bound MIANS was quantitated by the absorbance measurement at 322 nm ( $\epsilon = 17000 \text{ mol}^{-1} \cdot \text{cm}^{-1}$ )<sup>[14]</sup>.

#### 1.6 Fluorescence resonance energy transfer between MIANS-MRP1 and TNP-ATP (or TNP-ADP)

The fluorescence emission spectrum of MIANS-labeled MRP1 (50 μg/mL in buffer D) was recorded following excitation at 322 nm in the absence or presence of 30 μmol/L TNP-ATP (or TNP-ADP). The fluorescence emission spectrum of MIANS bound to MRP1 ( $\lambda_{\text{ex}} = 322 \text{ nm}$ ,  $\lambda_{\text{em}} = 420 \text{ nm}$ ) has a high degree of overlap with the excitation spectrum of TNP-ATP or TNP-ADP ( $\lambda_{\text{ex}} = 408 \text{ nm}$ ,  $\lambda_{\text{em}} = 535 \text{ nm}$ ). Therefore, the expectation that fluorescence resonance energy transfers from MIANS (the donor) and TNP (the acceptor) would take place if they are located at a suitable distance apart (<40 nm)<sup>[15]</sup>.

#### 1.7 Collisional fluorescence quenching of MIANS-MRP1 by quenchers

Stock solutions of 5 mol/L acrylamide, 5 mol/L KI, and 5 mol/L CsCl were all freshly prepared in buffer D, and 0.1 mmol/L Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> was added to KI stock solution to prevent I<sub>3</sub><sup>-</sup> formation. The quenchers as 5 μL aliquot in buffer D were added to 500 μL of 50 μg/mL MIANS-MRP1 in the same buffer. Parallel experiments were carried out using MIANS-DTE, to assess quenching of MIANS by the same agents when completely accessible in an aqueous solution. Acrylamide, I<sup>-</sup>, and Cs<sup>+</sup> quenching data were analyzed using the Stern-Volmer equation<sup>[16]</sup>:

$$F_0/F = 1 + Kq \tau_0 [Q] = 1 + K_{sv} [Q],$$

where  $F_0$  and  $F$  are fluorescence intensities in the absence or presence of quencher, respectively,  $Kq$  is the bimolecular quenching constant,  $\tau_0$  is the fluorescence lifetime in the absence of quencher,  $[Q]$  is the concentration of quenching agent, and  $K_{sv}$  is the Stern-Volmer quenching constant. A Stern-Volmer plot of  $F_0/F$  vs  $[Q]$  gives a linear plot with slope of  $K_{sv}$ .

## 2 Results

### 2.1 Purification of MRP1

The purified MRP1 protein was subjected to SDS-PAGE (7.5% polyacrylamide gel) and detected by silver staining (fig. 1(a), lane 2) or Western blot

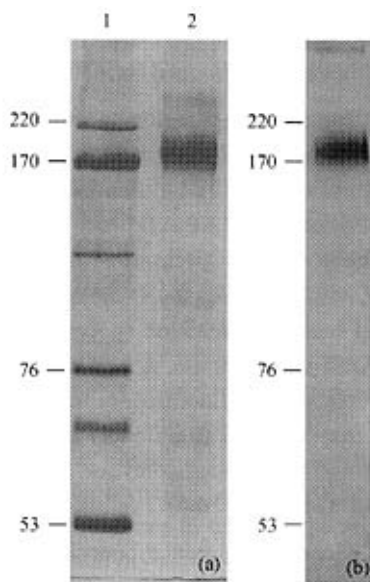


Fig. 1. Purification of MRP1 from BHK cells transformed by cDNA of MRP1/His10. The sample was dialyzed against a buffer (50 mmol/L Tris-HCl, pH 7.4, 2 mmol/L CHAPS, 0.15 mol/L  $\text{NH}_4\text{Cl}$ , 1 mmol/L DTT) and then analyzed by SDS-PAGE (7.5%) with silver staining. (a) Lane 1, markers; lane 2, 0.3  $\mu\text{g}$  purified MRP1; (b) Western blot (antibody MRP1) of lane 2 in (a).

analysis with MRP1 antibody (fig. 1(b)). Greater than 90% of the purified proteins were MRP1 protein as judged by densitometry of a silver-stained SDS-PAGE. The result would be available for further study the anti-drug mechanism of MRP1.

### 2.2 Effects of sulfhydryl-reactive agents on MRP1 ATPase activity

ATP was hydrolyzed in proportion to the amount of the purified MRP1 protein assayed, and typical Michaelis-Menten behavior was exhibited, yielding estimations of  $K_m = 2.8 \text{ mmol/L}$  and  $V_{max} = 110 \text{ nmol P}_i \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ . Fig. 2(a) shows that the ATPase activity of the purified MRP1 was inhibited in a concentration-dependent fashion by treatment with MIANS or NEM, and the inhibition of MIANS is greater than that of NEM. Various anticancer drugs can activate MRP1 ATPase activity to different degree (fig. 2(b)).

### 2.3 Effects of nucleotides and drugs on fluorescence labeling of MRP1 with MIANS

MIANS only becomes fluorescence after the maleimide moiety undergoes covalent reaction with sulfhydryl groups<sup>[17]</sup>. This feature allows the time-course of labeling to be conveniently followed by continuous monitoring of the fluorescence change. Addition of MIANS to the purified MRP1 (50  $\mu\text{g/mL}$ ) led

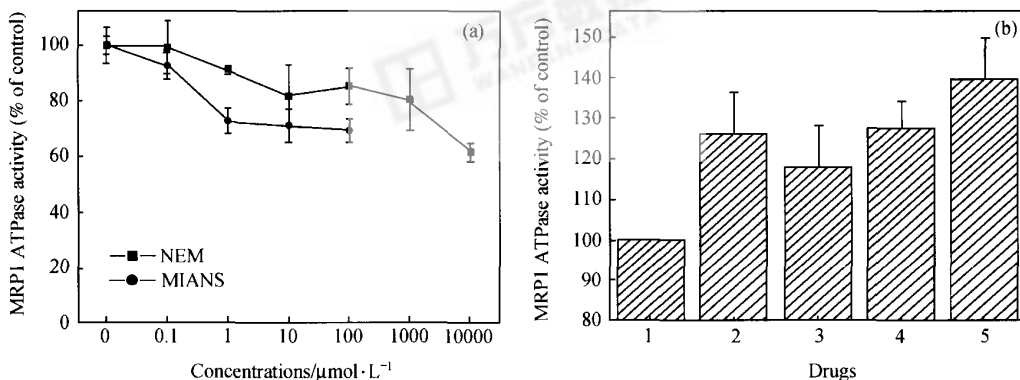


Fig. 2. Effects of various compounds on MRP1 ATPase activity. The activity was represented as percentage of the control without any compounds. (a) The concentration-dependence of inactivation of MRP1 ATPase activity by MIANS or NEM. Purified MRP1 was treated with increasing concentrations of MIANS or NEM for 1 h at 22°C, and the ATPase activity was determined after quenching of unreacted MIANS with DTE. (b) Effects of anticancer drugs on MRP1 ATPase activity: 1, none; 2, 4  $\mu\text{mol/L}$  vincristine; 3, 4  $\mu\text{mol/L}$  vinblastine; 4, 4  $\mu\text{mol/L}$  daunorubicin; 5, 0.3 mmol/L GSSG.

to a rapid increase in fluorescence emission intensity (fig. 3(a)), the rate of increase in fluorescence declined smoothly over a period of about 30 min. As shown in fig. 3(a), the fluorescence intensity of MRP1 labeled with MIANS was enhanced gradually when the concentration of MIANS increased, and it reached the highest when concentration of MIANS was 4  $\mu\text{mol/L}$ . The result indicated that the fluorescence labeling of MRP1 with MIANS was saturable, therefore, 4  $\mu\text{mol/L}$  MIANS was used in the following experiments unless otherwise stated.

The MIANS probe is known to be highly sensitive to the polarity of its immediate surroundings<sup>[17]</sup>, with the emission maximum exhibiting a blue shift in a nonpolar environment. The fluorescence emission spectrum of MIANS-labeled MRP1 is shown in fig. 3(b), compared to the spectrum for MIANS following reaction with the soluble sulfhydryl compound DTE. The emission maximum for MIANS-DTE (Curve 2) is at about 450 nm, whereas that for MIANS-MRP1 (Curve 3) is at about 420 nm. Such a large blue shift indicates that the labeled Cys residues in MRP1 reside in a relatively hydrophobic environment.

Purified MRP1 (50  $\mu\text{g/mL}$  in 500  $\mu\text{L}$  buffer D) was preincubated with various concentration of ATP (or ADP), and then labeling was initiated by addition of MIANS to a final concentration of 4  $\mu\text{mol/L}$ . Preincubation with ATP (or ADP) resulted in a reduction

in the rate of reaction of MRP1 with MIANS, which was dependent on the ATP (or (ADP), the plots were omitted) concentration added (fig. 4(a)). The rate of reaction of MRP1 with MIANS also reduced if the purified MRP1 was preincubated with various drugs, GSH and GSSG (fig. 4(b)). If MRP1 was first reacted with NEM (fig. 4(a)), no increase in fluorescence intensity was observed after the addition of MIANS, indicating that the two agents compete for reaction with the same sites on the transporter.

#### 2.4 Stoichiometry of MIANS labeling of MRP1

The experimental results above showed that a substantial change took place in fluorescence properties of MIANS after it reacted with MRP1, which indicated that the Cys residues on MRP1 could be modified by MIANS. To measure the stoichiometry of MIANS labeling, 1  $\mu\text{mol}$  MRP1 was treated with increasing concentration of MIANS. The experimental results (fig. 5) indicated that the fluorescence intensity of MIANS-labeled MRP1 did not further increase when the concentration of MIANS reached 4  $\mu\text{mol/L}$ . The actual concentration of MIANS reacted with MRP1 was determined using absorbance measurements. The absorbance of 1  $\mu\text{mol/L}$  MIANS at an excitation wavelength of 322 nm should be 0.017 ( $\epsilon = 17000 \text{ mol}^{-1} \cdot \text{cm}^{-1}$ )<sup>[14]</sup>. The experimental results indicated that the absorbance value of MIANS react-

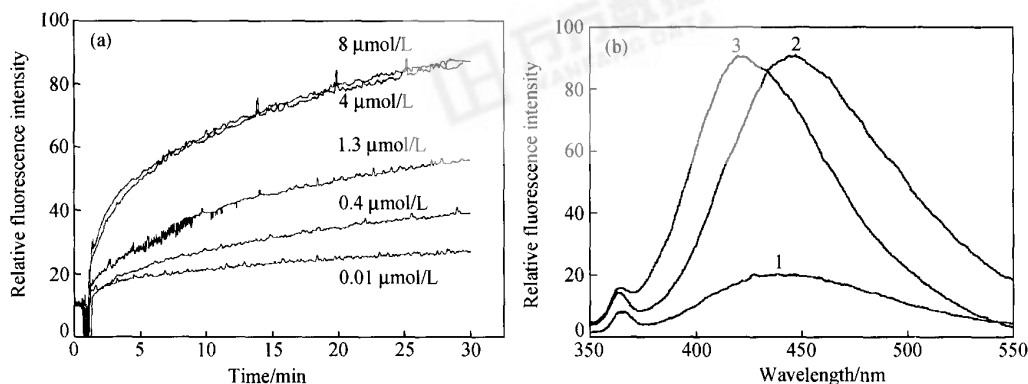


Fig. 3. Fluorescence change of MRP1 labeled with MIANS. (a) MRP1 incubated with increasing concentrations of MIANS and the fluorescence change monitored using an F-4010 fluorescence spectrophotometer. Saturable titration indicates that a limited amount of cysteine residues on MRP1 are accessible to MIANS. (b) Fluorescence emission spectra of MIANS alone and MIANS-MRP1 complex and a soluble compound DTE. Curve 1, 4  $\mu\text{mol/L}$  MIANS ( $\lambda_{em}=440 \text{ nm}$ ); Curve 2, 4  $\mu\text{mol/L}$  MIANS reacted with DTE ( $\lambda_{em}=450 \text{ nm}$ ); Curve 3, 4  $\mu\text{mol/L}$  MIANS reacted with MRP1 ( $\lambda_{em}=420 \text{ nm}$ ).

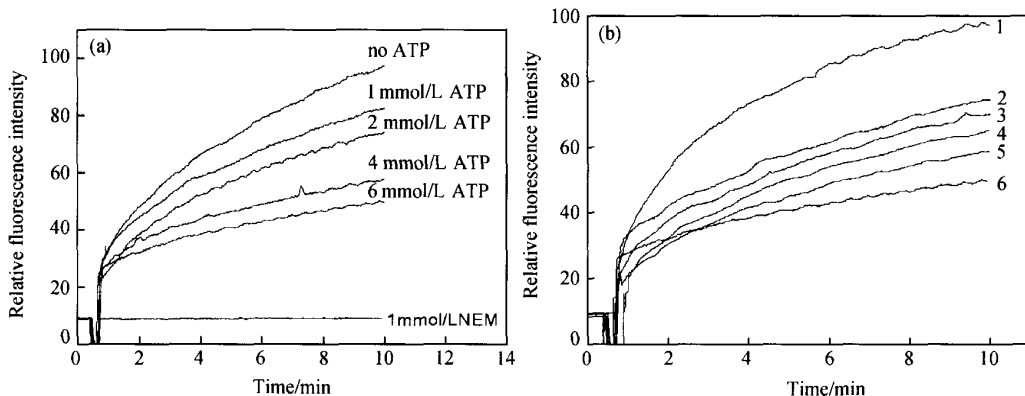


Fig. 4. The effects of nucleotides and drugs on the reaction of MRP1 with MIANS. (a) MRP1 was labeled with 4  $\mu\text{mol/L}$  MIANS in the presence of various concentrations of ATP (or ADP (the plot is omitted)). (b) MRP1 was labeled with 4  $\mu\text{mol/L}$  MIANS in the presence of various drugs. 1, none; 2, 0.3 mmol/L GSSG; 3, 0.3 mmol/L GSH; 4, 4  $\mu\text{mol/L}$  daunorubicin; 5, 4  $\mu\text{mol/L}$  vincristine; 6, 4  $\mu\text{mol/L}$  vinblastine.

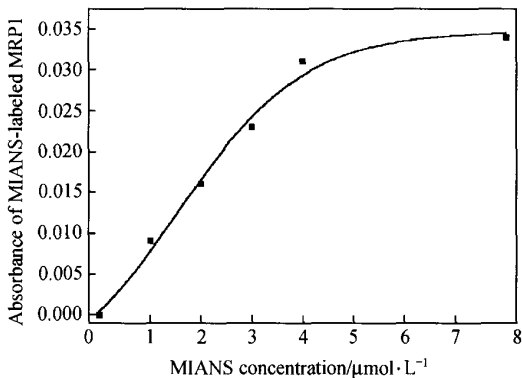


Fig. 5. Stoichiometry of MIANS labeling of MRP1. Purified MRP1 was treated with the increasing concentration of MIANS. Following quenching of the unreacted MIANS with DTE, dialysis, and lyophilization, the labeled MRP1 samples were redissolved in 6 mol/L guanidine hydrochloride. The amount of the bound MIANS to MRP1 was quantitated by absorbance measurement at 322 nm.

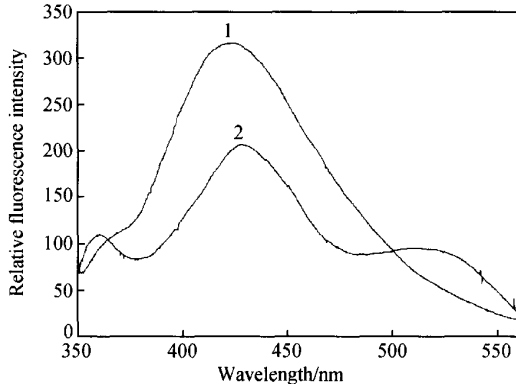


Fig. 6. Fluorescence resonance energy transfers between MIANS-MRP1 and TNP-ATP. Fluorescence emission spectrum of MIANS-MRP1 (50  $\mu\text{g/mL}$ ) was excited at 322 nm in the absence (Curve 1) or presence (Curve 2) of 30  $\mu\text{mol/L}$  TNP-ATP (or TNP-ADP (the plot was omitted)).

ing fully with MRP1 (1  $\mu\text{mol}$ ) is 0.035, which demonstrated that mole ratio of MIANS to MRP1 is 2/1.

2.5 Cys residues of MRP1 modified by MIANS lie close to its NBDs

As mentioned above, the fluorescence emission spectrum of MIANS-MRP1 has a high degree of overlap with the excitation spectrum of TNP-ATP or TNP-ADP. Therefore, fluorescence resonance energy transfer from MIANS (the donor) to TNP (the acceptor) would take place. Fig. 6 shows that a fluorescence emission was observed at 535 nm when MIANS-MRP1 was excited at 322 nm in the presence of

30  $\mu\text{mol/L}$  TNP-ATP(or TNP-ADP), which indicated that energy transfer between the MIANS and TNP-ATP (or TNP-ADP) took place. Lakowicz<sup>[18]</sup> had reported that fluorescence resonance energy transfer from the donor to the acceptor can only take place if they are located a suitable distance apart (<40 nm)<sup>[18]</sup>. These results showed that the residues labeled by MIANS are located close to the TNP-ATP (or TNP-ADP (the plot is omitted)) binding domains (NBDs).

2.6 The fluorescence quenching of MIANS-MRP1 caused by quenchers

Fluorescence quenching has proved to be a very

useful technique to provide information on polarity and charge of the region in the vicinity of the bound probe. The collisional quenchers, acrylamide,  $\Gamma^-$ , and  $\text{Cs}^+$  were used to assess the solvent accessibility and local environments of MIANS bound to MRP1. In each case, a parallel experiment was performed with MIANS bound to a small soluble molecule DTE. As shown in fig. 7(a), MIANS bound to MRP1 (Curve 1)

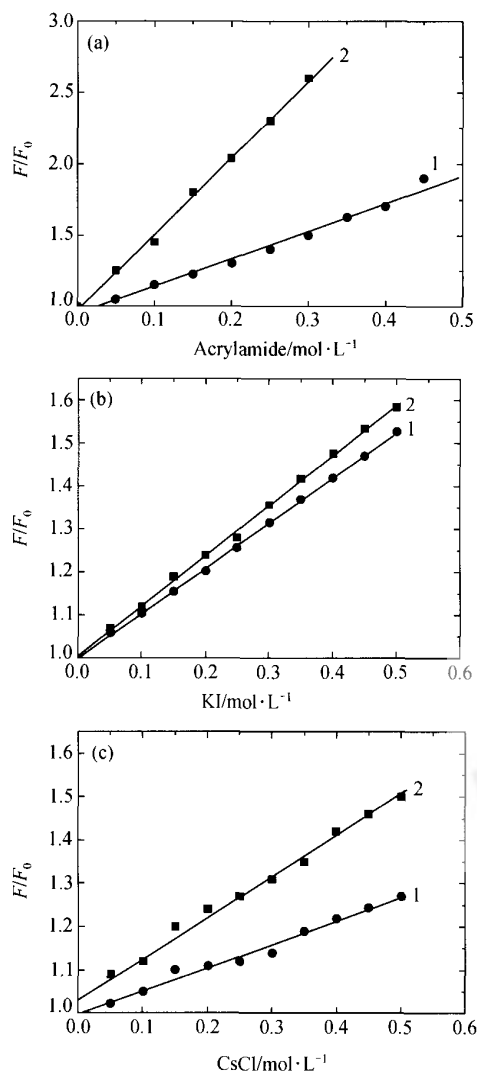


Fig. 7. Stern-Volmer plots for quenching of MIANS-MRP1 and MIANS-DTE by (a) acrylamide, (b) iodide ion  $\Gamma^-$ , and (c)  $\text{Cs}^+$ . Parallel experiments were carried out using MIANS-MRP1 ( $\bullet$ , Curve 1) and MIANS-DTE ( $\blacksquare$ , Curve 2).

was quenched poorly by acrylamide, compared to the quenching observed for the MIANS bound to DTE (Curve 2), where it is completely accessible in aqueous solution. This finding shows that the probe is protected from the external environment when bound to the cysteine residues on MRP1, likely because it is buried within the protein molecules.  $\text{Cs}^+$  (fig. 7(c)) was also a poor quencher of MIANS fluorescence, whereas  $\Gamma^-$  (fig. 7(b)) was a highly effective quencher of MIANS-MRP1, which was quenched similarly to MIANS-DTE.

### 3 Discussion

MRP1 contains three transmembrane domains (TMDs), which is in charge of recognizing and binding with the drugs and substrates, and two nucleotide binding domains (NBDs) with the characteristic of members of the ATP-binding cassette superfamily<sup>[6]</sup>. Human MRP1 contains a total of 25 Cys residues, and it is important for elucidating the anti-cancer mechanism of MRP1 and understanding the function of these Cys residues. Our experimental results by using MIANS, a probe reacting specially with Cys residues in protein, demonstrated that the emission maximum for MIANS-MRP1 (fig. 3(b), Curve 3) exhibits a 30 nm blue shift, compared to that of MIANS-DTE (fig. 3(b), Curve 2). Such a large blue shift indicates that the labeled Cys residues in MRP1 reside in a relatively hydrophobic environment. At the same time, there is a substantial decrease in the rate of labeling was observed (fig. 4(a)) when MRP1 was incubated with MIANS in the presence of ATP (or ADP). These results suggested that the Cys residues on MRP1 modified by MIANS located in the vicinity of its NBDs, which was confirmed by the results obtained by the experiment of fluorescence resonance energy transfer (fig. 6). Only two Cys residues in MRP1 can react with MIANS (fig. 5), furthermore, a fluorescence resonance energy transfer takes place between not only MIANS-MRP1 and TNP-ATP but also MIANS-MRP1 and TNP-ADP (fig. 6). In view of ATP binding exclusively at NBD1<sup>[9]</sup> and ADP trapping predominantly at NBD2<sup>[11]</sup>, it might suggest that one Cys

residue on MRP1 modified by MIANS may lie in the vicinity of NBD1 and the other may be located in the vicinity of NBD2. The results obtained with the quenchers suggest that the labeled Cys residues in MRP1 reside in a relatively hydrophobic environment, and that the region around the MIANS-labeled cysteine is positively charged. These results can be used to explain rationally why the NBDs are easy to be bound with negatively charged nucleotides. But our results did not confirm which Cys residues in MRP1 are modified by MIANS at the moment. It is obvious that some site-directed mutation experiments should be further carried out to solve the problem.

Normally, transmembrane domains (TMDs) in MRP1 take charge of recognizing and binding with the drugs and substrates<sup>[19–22]</sup>. The experimental results demonstrate that anticancer drugs, such as vincristine, vinblastine, daunorubicin, can not only reduce the rate of MIANS labeling of MRP1 (fig. 4(b)) but also affect the MRP1 ATPase activity (fig. 2(b)). There is still no direct evidence so far to prove that anticancer drugs can occupy the NBDs that are believed to bind to nucleotides. A reasonable explanation of our experimental results is that binding of drugs to the TMDs of MRP1 may make a change in conformation of the protein, which in turn alters the accessibility of the two Cys residues bound by MIANS and further affect MRP1 ATPase activity (fig. 2(b)) and the coupling with the transport of its substrates. Hydrolysis of ATP provides energy for MRP1 to translocate actively the drugs. Because it is not easy to obtain enough purified MRP1, there are few reports involved in the mechanism on the coupling of translocation of the transported species with hydrolysis of ATP in the protein. In this paper, the experimental results about the interaction of TMDs and NBDs, the relationship between the conformation change and ATPase activity of MRP1 obtained by using the MIANS, a sulfhydryl-reactive fluorescent probe, have provided direct evidence for the coupling of translocation of the transported species with hydrolysis of ATP in MRP1.

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