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Conserved Walker A Cysteines 431 and 1074 in Human P-glycoprotein Are Accessible to Thiol-Specific Agents in the Apo and ADP-Vanadate Trapped Conformations

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Abstract

P-glycoprotein (P-gp) is an ATP-binding cassette efflux transporter involved in the development of multidrug resistance in cancer cells. Although the mechanism of P-gp efflux has been extensively studied, aspects of its catalytic and transport cycle are still unclear. In this study, we used the conserved C431 and C1074 in the Walker A motif of nucleotide-binding domains (NBDs) as reporter sites to interrogate the interaction between the two NBDs during the catalytic cycle. Disulfide crosslinking of the C431 and C1074 residues in a cystless background can be observed in the presence of M14M and M17M crosslinkers, which have spacer arm lengths of 20 Å and 25 Å, respectively. However, crosslinking with both crosslinkers was prevented in the ADP-vanadate trapped (closed) conformation. Both C431 and C1074 alone or together (double mutant) in the apo and closed conformations were found to be accessible to fluorescein-5-maleimide (FM) and methanethiosulfonate derivatives of rhodamine and verapamil. In addition, C1074 showed 1.4-fold and 2-fold higher degrees of FM labeling compared to C431 in the apo and closed conformations, respectively, demonstrating that C1074 is more accessible than C431 in both conformations. In the presence of P-gp substrates, crosslinking with M17M is still observed, suggesting that binding of substrate in the transmembrane domains does not change the accessibility of the cysteines in the NBDs. In summary, the cysteines in the Walker A motifs of NBDs of human P-gp are differentially accessible to thiol-specific agents in the apo and closed conformations.

Introduction

The ATP-binding cassette (ABC) superfamily constitutes one of the largest families of transport proteins.¹ Of the 48 human ABC proteins, 17 are implicated in diseases.² P-glycoprotein (P-gp, ABCB1) is a clinically important ABC transporter that is involved in the development of multidrug resistance (MDR). Powered by the hydrolysis of ATP, P-gp is able to efflux various structurally dissimilar and functionally divergent chemotherapeutic drugs. Although the mechanism of efflux by P-gp has been extensively studied, there are still aspects of the catalytic and transport cycle that are not well understood.

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P-gp is a single polypeptide consisting of 1280 amino acids, with two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). While the NBDs have conserved motifs across species, the TMDs show little sequence homology. There are still unanswered questions concerning the molecular details as to how ATP hydrolysis is coordinated between the two NBDs during a catalytic cycle. The current consensus is that there are three distinct conformations during one catalytic cycle of P-gp. In the absence of a transport substrate or a nucleotide, P-gp exists in the apo conformation, which is a 'high affinity inward-facing orientation' (in the shape of an inverted 'V'). This conformation is also supported by the recent X-ray crystal structures of mouse P-gp³ and *Caenorhabditis elegans* P-gp.⁴ Preceding hydrolysis, there is evidence for the formation of a reaction intermediate (E•S) that can be obtained using ATP- γ -S, a non-hydrolyzable analog of ATP.⁵ In this conformation which is temperature-dependent (occurs at 34 °C but not 4 °C), the ATP- γ -S is occluded into the P-gp NBDs. The third conformation is the ADP-vanadate (V_i) trapped post-hydrolysis (E•P) conformation which assumes an outward-facing orientation (in the shape of a regular 'V'). In the ATP- γ -S occluded and the ADP-V_i trapped closed conformations there is a reduced affinity for drug substrates.⁵

The X-ray crystal structure of P-gp in the closed conformation (in the presence of bound nucleotide) has not yet been reported. Therefore, the study of the conformational changes occurring during the catalytic cycle of P-gp is elucidated mainly by biochemical assays. Studies using disulfide crosslinking between cysteine residues in the TMDs and NBDs of P-gp in different conformations has been extensively used as a strategy to characterize these sites.^{6, 7} Human wild-type (WT) P-gp contains a total of seven cysteine residues, with two cysteines (C431 and C1074) in the Walker A motif of NBDs. When all the cysteines are mutated to alanines with site-directed mutagenesis, the cysteine-less (cysless) WT P-gp is still fully functional.⁸ Therefore, cysteine residues are not essential for ATP hydrolysis or drug transport.

As C431 and C1074 line the ATP pocket of the NBDs, they are excellent sites for covalent modification by thiol-reactive probes such as fluorescein-5-maleimide (FM) and methanethiosulfonate (MTS) conjugated compounds. A more complete understanding of the accessibility of these native cysteines to thiol-specific probes would be useful to study conformational changes of P-gp and allow the mapping of distance changes in the NBDs during its catalytic cycle.

In this study, we determined the accessibility of conserved C431 and C1074 by disulfide crosslinking and changes in fluorescence labeling, using homo-bifunctional crosslinkers of various spacer arm lengths and FM, respectively. We also investigated the accessibility of these cysteines in the presence of the transport substrate, cyclosporine A (CsA). Here, we report the distance between the two NBDs of human P-gp to be 20–25 Å as the C431-C1074 distance, which is less than the reported distance in the X-ray crystal structure of mouse P-gp (36 Å) and *C. elegans* P-gp (54 Å). C431 and C1074 are accessible to the thiol-reactive compounds in both the apo (open) and ADP-V_i trapped (closed) conformations, with C1074 being more accessible to 6 FM than C431. Accessibility of both the cysteines in the NBDs is not affected by the binding of the transport substrate (CsA) in the TMDs. This accessibility in both conformations of P-gp can be due to a change in the orientation of the cysteines from inward-facing to outward-facing within the ATP pocket. Thus, our results demonstrate that the cysteines in the Walker A motifs in NBDs of human P-gp are differentially accessible in the apo and closed conformations.

Experimental Procedures

Chemicals

Homobifunctional crosslinkers M8M (3,6-dioxaoctane-1,8 diylbis-methanethiosulfonate), M14M (3,6,9,12-tetraoxatetradecane-1,14-diylbismethane thiosulfonate), M17M (3,6,9,12,15-pentaoxaheptadecane-1,17-diylbismethane thiosulfonate), MTS-rhodamine and MTS-verapamil were obtained from Toronto Research Chemicals, Inc. (Downsview, ON, Canada), cyclosporine A was purchased from Calbiochem (San Diego, CA), fluorescein-5-maleimide (FM) was obtained from Invitrogen (San Diego, CA). The P-gp-specific monoclonal antibody C-219 was obtained from Fujirebio Diagnostics Inc. (Malvern, PA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Construction of mutants

Histidine-tagged cysless wild type (WT) P-gp was constructed by replacing the seven endogenous cysteines at positions 137, 431, 717, 956, 1074, 1125 and 1227 with alanines. Cysteine residues were re-introduced at 431 and 1074 individually or together into the NBDs of a histidine-tagged cysless P-gp cDNA as described previously.⁹ In addition, N611C and T1256 were replaced individually or together with cysteine residues in a cysless background.

Preparation of crude membranes from High-Five insect cells infected with recombinant baculovirus carrying the WT or mutant human MDR1 gene

High-Five insect cells (Invitrogen, Carlsbad, CA) were infected with the baculovirus carrying the human MDR1 cDNA (cysless WT, C431, C1074, C431/C1074, N611C, T1256C and N611C/T1256C) with a 6X histidine tag at the C-terminal end as described previously.¹⁰ Crude membranes were prepared and stored at -80°C , as described previously.¹¹

Cell lines

HeLa cells were cultured in DMEM media supplemented with 10% FBS, 1% glutamine, and 1% penicillin and maintained as described previously.¹²

Transduction of HeLa cells with Bac-Mam baculovirus

The BacMam-Pgp virus carrying the human MDR1 cDNA (cysless WT, C431, C1074, C431/C1074, N611C, T1256C and N611C/T1256C) was added to HeLa cells (2.5 million) at a titer of 50 to 60 viral particles per cell in 3 ml of DMEM and was incubated for 1 h at 37°C . Up to 20 ml of DMEM was added and the cells were further incubated for 3 to 4 h. A total of 10 mM butyric acid was then added, and the cells were incubated for another 20 h at 37°C . After 24 h, the cells were trypsinized, washed, counted, and analyzed by flow cytometry for cell surface expression and function. For cell surface detection of P-gp, the human P-gp-specific monoclonal antibody MRK-16 was used and fluorescence of FITC was measured on a FACSort flow cytometer equipped with a 488 nm argon laser and 530 nm bandpass filter. To assess the transport function, rhodamine 123 (1.3 μM), NBD-cyclosporin A (0.5 μM), daunorubicin (0.5 μM) and calcein-AM (0.5 μM) were used, as described previously.¹²

Vanadate-sensitive ATPase assay

The ATPase activity of P-gp in insect cell crude membranes was measured by the end-point inorganic phosphate (P_i) assay as previously described.¹³ P-gp-specific activity was recorded as the V_i -sensitive ATPase activity. Crude membranes (10 – 20 μg protein/100 μL) from High-Five insect cells expressing the cysless WT or cysteine mutations of P-gp were

incubated under different conditions with varying concentrations of compounds under investigation (FM, MTS-verapamil, MTS-rhodamine) in the presence and absence of sodium orthovanadate (0.3 mM) in ATPase assay buffer (100 mM MES-Tris pH 6.8, 100 mM KCl, 10 mM NaN₃, 2 mM EGTA, 2 mM Ouabain, 20 mM MgCl₂, H₂O). The reaction was initiated by the addition of 5 mM ATP and incubated for 20 min at 37 °C. SDS solution (0.1 mL of 5% SDS) was added to terminate the reaction, and the amount of P_i released was quantified with a colorimetric reaction, as described previously.¹³

Disulfide crosslinking with homobifunctional methanethiolsulfonate (MTS) crosslinkers and MTS-reagents

Membranes prepared from High-Five insect cells (50 µg protein in 50 µL of buffer [10 mM Tris-HCl, 150 mM NaCl, pH 7.5]) expressing C431 and/or C1074 as well as N611C and/or T1256C mutant P-gps in the absence of added drug-substrate or ATP (apo conformation) were treated with the desired homobifunctional disulfide crosslinkers (M8M, M14M or M17M) at 200 µM for 15 min. at 4 °C. To generate the ADP-V_i (closed) conformation, the membranes were first treated with 0.3 mM sodium orthovanadate, 10 mM MgCl₂, 5 mM ATP at 37 °C for 20 min, the samples were chilled at 4 °C for 5 min and then subsequently treated with desired homobifunctional disulfide crosslinkers (200 µM) (M8M, M14M or M17M) for 15 min. at 4 °C. The reactions were stopped by the addition of 5 X SDS PAGE sample buffer without reducing agent, β-mercaptoethanol (β-ME). The samples were subjected to SDS/PAGE (7% Tris-Acetate gel, Invitrogen) at a constant voltage of 150 V for 2 hours and immunoblot analysis was performed with P-gp specific mouse monoclonal antibody C219 and enhanced chemiluminescence (ECL, GE healthcare) as per the manufacturer's protocol. For crosslinking with MTS-reagents (MTS-verapamil and MTS-rhodamine), High-Five insect cell membranes expressing cystless WT, C431, C1074, C431/C1074 (20 µg protein in 95 µL of ATPase assay buffer) in apo conformation were treated with MTS-verapamil or MTS-rhodamine at 10 µM for 20 min at 4 °C. After the crosslinking reaction, 5 mM ATP (5 µL, 100 mM stock) is added to the reaction mixture and the ATPase activity is evaluated as described previously.

Labeling with fluorescein-5-maleimide and fluorescence spectroscopy

Fluorescein-5-maleimide was used to label the P-gp single and double cysteine mutants in both the apo and closed conformations. Briefly, 100 µg protein/50 µL of crude membranes from the High-Five insect cells expressing cystless WT and C431/C1074 and N611C/T1256C (single or double cysteine mutants) in the apo, Mg²⁺-ATP and Mg²⁺-ATP plus 0.3 mM V_i to generate ADP-V_i (closed conformation) were incubated with 100 µM of FM in 2 % DMSO for 5 mins at 37 °C in the dark (The level of P-gp protein in insect cell crude membranes is ~2%. For labeling with FM, 0.28–0.30 µM of P-gp and the molar fold excess of FM concentration was 330). To generate the closed conformation, the crude membranes were first incubated with 0.3 mM V_i, 20 mM MgCl₂ and 10 mM or 20 mM ATP and incubated for 10 mins at 37 °C. To make sure the cysteine mutants remained in the closed conformation before crosslinking with FM, 10 µg of protein sample that was treated with ATP and V_i to generate ADP-V_i (closed) conformation was assessed for ATPase activity. Following this, 100 µM of FM was added for 5 mins at 37 °C in the dark. The reaction was then quenched with 5 µL of 500 mM EDTA and 12.5 µL of 5X SDS page sample buffer. For immunoprecipitation of FM-labeled P-gp, 700 µL RIPA buffer and 10.5 µg antibody C219/100 µg of P-gp was added, and the reaction mixture was incubated at room temperature for 30 min in the dark with rocking. Protein A sepharose beads (35 µL) in RIPA buffer were added and further incubated for 2.5 hours with rocking in the dark. The beads were pelleted by centrifugation at 13,000 rpm for 5 min. at 4 °C. The beads were washed 3 times with RIPA buffer (800 µL), and twice with 800 µL of 50 mM Tris-HCl (pH 7.5) added with 500 mM NaCl. 2X SDS-PAGE sample buffer (25 µL) containing no β-ME was added,

followed by incubation at 42 °C for 30 min. A further 25 µL of de-ionized water was added, and the mixture was incubated at 42 °C for another 30 minutes before samples were subjected to SDS-PAGE (7 % Tris-acetate gel) at a constant voltage of 150 V for 1.5 hours. The gel was scanned for blue fluorescence and the fluorescence incorporated into the P-gp band was quantified using the STORM 860 BlueFluorescence Imager system (Molecular Dynamics, 11 Sunnyvale, CA) and the software ImageQuaNT. The fluorescence incorporated into cystless WT and C431/C1074 P-gp is quantified by the software ImageQuaNT and then corrected for background fluorescence in the respective lanes. The expression levels of P-gp in the cysteine mutants were determined by colloidal blue staining of the same gel that was used for fluorescence imaging and quantification. The protein levels of mutant P-gps (C431, C1074 and C431/C1074) are expressed as a ratio relative to cystless WT by quantifying the amount of colloidal blue stained mutant P-gps and normalizing it to the amount of cystless WT. The final level of fluorescence (in arbitrary units) was calculated as (FM Fluorescence incorporated into P-gp mutant – background fluorescence)/(ratio of mutant P-gp expression relative to cystless WT protein).

Molecular Modeling

A homology model of human Pgp in an open, inward-facing conformation was built on the basis of the X-ray structure of QZ59-RRR-bound mouse Pgp (3G60.pdb).³ The model was generated using only one molecule (chain A) of the two present in the crystal asymmetric unit. The sequence alignment was done by BLAST.¹⁴ The model was constructed by *in-silico* mutation of the protein residues to the human Pgp sequence, and three residues (D87-L88-M89, absent in the mouse mdrla-Pgp sequence) were manually modeled at extracellular loop 1 (ECL1). Finally, side-chain conformations of the new residues were visually inspected and modified when necessary. The stereochemical quality of the models was inspected by PROCHECK;¹⁵ some fragments of the structures were submitted to geometrical regularization using Coot¹⁶ to improve the stereochemical quality. The final structure had only 2 close contacts (A198-O to T202-OG1 = 2.18 Å and P66-CB to T202-OG1 = 2.19 Å) and a Ramachandran plot showed 73.7% residues in most favored regions, 19.5% in additional allowed regions, 6.8% in generously allowed regions and no residues in disallowed regions.

The homology model of human P-gp in the closed, outward-facing conformation was assembled with the NBD domains built on the basis of the Hly-B structure, as described in Ohnuma et al¹⁷ and the TMD constructed by O'Mara & Tieleman, on the basis of the *Staphylococcus aureus* Sav1866 structure. The NBDs and TMDs fit together very well and the joint areas were regularized and carefully inspected using Coot;¹⁶ the emerging model was finally energy-minimized by CHARMm.¹⁸

Docking studies of FM at the NBDs of the homology model of human Pgp based on the X-ray structure of Hly-B were carried out using the program AutoDock Vina.¹⁹ The receptor and ligand structures were prepared with the MGLtools software package.²⁰ Docking jobs were carried out in the free ATP site model, at the ATP site-1 with the K433 side-chain set as flexible, the search box centered at the γ -phosphate of the original ATP molecule ($x=-2.295$ $y=5.702$ $z=-4.443$) and of 16Å×12Å×18Å dimensions; and at the ATP site-2 with the K1076 side-chain set as flexible, the search box centered at γ -phosphate of the original ATP molecule ($x=1.499$ $y=-6.416$ $z=12.957$) and of 20Å×20Å×18Å dimensions. Docking jobs were also run in the model with ATP, at the ATP site-1 with C431 in an outward-facing orientation using a rotamer taken from the library of Pymol, the search box centered at the sulfur atom of C431 ($x=1.734$ $y=10.9$ $z=-8.889$) and of 30Å×10Å×16Å dimensions; and at the ATP site-2 with C1074 in an outward-facing orientation using a rotamer taken from the

library of Pymol, the search box centered at the sulfur atom of C1074 ($x=7.63$ $y=-8.705$ $z=17.29$) and of $18\text{\AA}\times 28\text{\AA}\times 24\text{\AA}$ dimensions.

Results

Homology models of human P-glycoprotein

The model of full-length human P-gp in the apo form (open conformation) was built on the basis of the X-ray structure of mouse P-gp,³ while the model of human P-gp in the nucleotide-bound form (also called closed conformation) was built using the TMD-model built by Megan O'Mara and Peter Tieleman²¹ on the basis of the structure of Sav1866²² and the NBD-model based on the structure of Hly-B²³ (Figure 1). The Hly-B structure was chosen for these studies because it was crystallized with ATP/Mg²⁺, while the Sav1866 structure was crystallized in the presence of ADP and Na⁺.

Walker A C431 and C1074 located in NBD1 and NBD2, respectively, were selected for distance measurements using disulfide crosslinkers. This pair of cysteine residues was selected as they are native cysteines lining the ATP sites of the NBDs. The other pair of cysteine residues selected in this study was N611C/T1256C and they are located on the surface of the NBDs, away from the ATP-binding sites. From these homology models, the predicted distances between C431 and C1074 in the apo and closed conformations are 36 Å and 32 Å, respectively. The predicted distances between N611C and T1256C in the apo and closed conformations are 56 Å and 39 Å, respectively (Figure 1).

Cell surface expression and transport of fluorescent substrates by C431/C1074 and N611C/T1256C mutant P-gps in HeLa cells

The cell surface expression of C431/C1074, measured by MRK-16 staining, was similar to cysless WT (100 % compared to cysless WT), while the cell surface expression of N611C/T1256C was 50 % lower compared to cysless WT (Figures 2A and 2B). Despite the somewhat lower cell surface expression of N611C/T1256C, both pairs of NBD cysteine mutants exhibited normal transport function (90 – 100 %) with P-gp 14 substrates, rhodamine 123, NBD-cyclosporine A, daunorubicin and calcein-AM. Figures 2C and 2D show typical histograms for transport function with Rh123 as a substrate for these two double cysteine mutants. The transport function of mutant P-gps tested with four commonly used fluorescent substrates is summarized in Table S1.

Disulfide crosslinking with homobifunctional crosslinkers of cysless WT and C431/C1074, N611C/T1256C mutant P-gps in apo and closed conformations

Crude membranes prepared from High-Five insect cells expressing C431/C1074 or N611C/T1256C in apo and closed conformations were treated with M8M, M14M and M17M (with spacer arm lengths of 13 Å, 20 Å and 25 Å, respectively). The results are shown in Figure 3. Figure 3A shows the positions of the crosslinked (X-linked) (upper band) and un-crosslinked P-gp (lower band) obtained in the apo state. Crosslinking with M14M and M17M occurs in the apo conformation of P-gp (Figure 3A), but not in the closed conformation (Figure 3B). With a shorter crosslinker (M8M), crosslinking is absent in both conformations (Figure 3A). In the presence of 5 mM DTT (a reducing agent), there is inhibition of crosslinking, indicating that crosslinking is mediated by disulfide bond formation (Figure 3C). We also found that the mobility of cysless WT and C431/C1074 mutant P-gp on the gel in the apo and ADP-Vi closed conformation in the absence of crosslinkers was not altered (data not shown). In cysless WT, as expected, there was no crosslinking in both tested conformations (Figure 3D). In addition, treatment of the single cys mutants C431 and C1074 with M17M did not result in crosslinking (data given in Figure S1). As a comparison, N611C/T1256C was treated with M17M. As shown in the Figure S2

there was no crosslinking. This result is consistent with the greater separation of N611C and T1256C with a predicted distance of 56 Å in the apo conformation compared to C431 and C1074 with a predicted distance of 36 Å from the homology model of human P-gp (Figure 1). The data in 15 Figure 3B demonstrate that C431 and C1074 in the apo conformation can come much closer (20 – 25 Å) than the predicted distance by homology models or the distance observed in the crystal structure of mouse P-gp.³

FM-labeling of cysless WT, C431, C1074, C431/C1074, N611C, T1256C, and N611C/T1256C in apo and closed conformations

Crude membranes prepared from High-Five insect cells expressing single (C431, C1074, N611 and T1256) and double (C431/C1074 and N611C/T1256C) cysteine mutants in both the apo and closed conformations were labeled with FM. A vanadate-sensitive ATPase assay confirmed that C431/C1074 remained in the closed conformation prior to crosslinking with FM (Figure S3, as its ATPase activity was inhibited > 90% in the presence of V_i). As seen from the fluorogram in Figure 4A, quantification of the FM-labeled P-gp showed that C431 is labeled to a lesser extent in both the apo and closed conformations compared to C1074. With C431 and C1074, there is a similar extent of FM labeling in both conformations. The extent of labeling in the double cysteine mutant is about twice that of the individual single cysteine mutants in both conformations, with fluorescence intensity contributed by FM. There were no significant differences observed between the two conformations with C431/C1074. These results suggest that there are differential accessibilities of C431 and C1074 in both conformations.

The quantification of the FM-labeled P-gp after normalization with P-gp expression for each mutant (Figure 4B) showed that the single cysteine mutants N611C and T1256C in both conformations are FM-labeled to similar extents. Similar to C431/C1074, the extent of FM-labeling in the double cysteine mutants, after normalization of protein expression, is about twice that seen with the individual single cysteine mutants in both the conformations. With N611C/T1256C, no significant difference was observed between the apo and the closed 16 conformations, suggesting the accessibilities of these single cysteine mutants to FM are similar in both the conformations. In cysless WT, as expected, there was only background labeling with FM (< 20%) as seen in both Figures 4A and 4B.

When the NBDs are super-saturated with ATP (20 mM and 20 mM $MgCl_2$) (closed conformation) (Figure 4C) prior to labeling with FM, labeling was observed to an extent similar to the apo conformation. This result shows that the C431 and C1074 residues are oriented in a direction that is still accessible for FM-labeling when the ATP is bound to both the NBDs.

Inhibition of ATPase Activity with FM, MTS-verapamil and MTS-rhodamine

FM is a thiol-reactive probe that reacts with cysteines and verapamil and rhodamine are substrates of P-gp. We wanted to test whether similar to FM, MTS-verapamil and MTS-rhodamine can also label the cysteine residues in the NBDs. For this, V_i -sensitive ATP hydrolysis of these cysteine mutants after cross-linking with MTS-verapamil and MTS-rhodamine was determined. With cysless WT, there is minimal inhibition of ATP hydrolysis (<20%) by FM, indicating FM had no effect on this activity (Figure 5). With C431 and C1074 single and double cysteine (C431/C1074) mutants that line the ATP pocket, the ATP hydrolysis activity was drastically inhibited with IC_{50} values of $1.1 \pm 0.2 \mu M$ for C431, $0.7 \pm 0.1 \mu M$ for C1074 and $0.63 \mu M$ for C431/C1074 (Figure 5A). These results indicate that the observed effect of FM was due to its interaction with these cysteine residues. The lower IC_{50} values (concentration to inhibit 50% ATPase activity in the presence of thiol-reactive probe) for C1074 compared to C431 in all cases indicated that C1074 was more accessible

to FM for modification compared to C431, consistent with labeling of these residues with FM (Figure 4).

When conjugated with a MTS moiety, MTS-verapamil and MTS-rhodamine are reactive towards the cysteine residues. The specificity of the Walker A cysteines towards thiol-reactive 17 probes was also confirmed with MTS-verapamil and MTS-rhodamine, which have different bulk and size compared to FM (Figures S4 and S5). With MTS-verapamil, a concentration-dependent biphasic effect on the ATPase activity of cysless WT, the single and double C431/C1074 mutants was observed. At a low concentration of less than 1–2 μ M, MTS-verapamil marginally stimulated ATP hydrolysis of cysless WT, the single and double C431/C1074 mutants (100 – 120% ATPase activity). This stimulatory effect might possibly be due to non-covalent interaction of MTS-verapamil with P-gp, with the dominant effect of the verapamil moiety functioning as a substrate versus the MTS moiety for covalent interaction with the cysteine residue(s). At concentrations above 2 μ M, there was inhibition of ATPase activity seen with all the cysteine mutants due to the covalent modification of the thiol group by MTS. There was no inhibition of ATPase activity observed for cysless WT, as expected.

With 10 μ M MTS-verapamil and MTS-rhodamine, there was inhibition of P-gp ATPase activity for single and double C431/C1074 mutants (ATPase activity is reduced to 20 – 40 % compared to basal activity of 100 % for MTS-verapamil (Figure S4) and ATPase activity is 50 – 80 % compared to basal activity for MTS-rhodamine (Figure S5).

With N611C or T1256C, the formation of a crosslinked FM-labeled product did not drastically inhibit the ATPase hydrolysis and the IC_{50} values of FM could not be determined for these mutants. The maximum ATPase inhibition by FM in N611C, T1256C and N611C/T1256C was 39 %, 11 % and 56 %, respectively (Figure S6).

Reversibility of covalent modification of C431, C1074 or C431/C1074 mutant P-gps by FM, MTS-verapamil and MTS-rhodamine

To test the reversibility of the disulfide-mediated covalent modification of the cysteine residues in the NBD mutants, 4 mM DTT was added to C431 and C1074 single and double cysteine C431/C1074 mutants after crosslinking with 10 μ M 18 MTS-verapamil and MTS-rhodamine. 10 μ M MTS-verapamil and MTS-rhodamine was chosen, as there is about 70% inhibition of ATPase activity in the NBD mutants. As shown in Figure S5, the ATPase activity of MTS-verapamil crosslinked NBD mutants was stimulated more than 3-fold compared to basal activity after the addition of 4 mM DTT. With MTS-rhodamine in the presence of 4 mM DTT, the ATPase activity was stimulated to a level higher than basal activity but the stimulation was not as high as in the case with MTS-verapamil. In both cases, stimulation of ATPase activity indicated the reversibility of ATPase activity by the breakage of the disulfide bond with 4 mM DTT. For FM-crosslinked C431/C1074 (Figure 5C), the presence of 4 mM DTT did not restore the ATPase hydrolysis of P-gp in either of the conformations, confirming the covalent thioether-mediated modification of the cysteine residues by the maleimide moiety.

Pretreatment with ATP protects C431, C1074 or C431/C1074 mutant P-gps from crosslinking with MTS-compounds and FM

As both C431 and C1074 line the ATP pocket, we hypothesized that protection of the pocket by pre-incubation with 5 mM ATP and 20 mM $MgCl_2$ would prevent FM from occupying the pocket and hence restore ATP hydrolysis. As expected, the ATPase activities of C431, C1074 and C431/C1074 were all protected from inactivation by FM by pretreatment with 5 mM ATP and 20 mM $MgCl_2$ prior to the addition of FM. The IC_{50} values of FM in these

single and double cysteine mutants could not be determined under these conditions, as the maximum inhibition of ATP hydrolysis of 38 %, 66 % and 68 % was observed for C431, C1074 and C431/C1074, respectively (Figure 5B).

For N611C and T1256C mutants, as expected, there was no change in the ATP hydrolysis profile with or without 5 mM ATP. In both cases, the IC_{50} values of FM in these single and double cysteine mutants could not be determined (Figure S6). In the presence of 5 mM ATP, the maximum inhibition of ATPase hydrolysis in these cysteine mutants was 26 %, 27 % and 51 % in N611C, T1256C and N611C/T1256C, respectively. These results were expected, since the cysteine mutants N611C and T1256C are on the surface of the NBDs of ATP and are fully accessible to covalent modification by FM even in the presence of ATP.

Preincubation with 10 mM ATP protects C431, C1074 single and double cysteine mutants from reaction with MTS-verapamil or MTS-rhodamine (10 μ M) by saturating the ATP pocket. As shown in Figure S5, the ATPase activity was retained at 50 – 90 % of basal activity as compared to the greater inhibition when the order of addition was reversed (MTS compounds followed by ATP). Comparing C431 with C1074, the ATPase activity of C431 is restored to a greater level than that achieved with C1074. This result confirms the greater accessibility of C1074 for covalent modification compared to C431.

Pretreatment with drug substrates does not inhibit crosslinking of C431/C1074 mutant P-gp with FM and M17M

Disulfide crosslinking is seen in C431/C1074 with the M17M crosslinker in the apo conformation. To determine whether binding of transport substrates in the drug-binding pocket in the TMDs,^{7, 24} has any effect on the accessibility of C431 and C1074 for crosslinking with thiol-reactive agents, C431/C1074 were first treated with substrates CsA (50 μ M), QZ 59 SSS (20 μ M) and actinomycin D (200 μ M) and then with M17M. These results are given in Figure S7 and the immunoblot in this figure demonstrates that pretreatment with a transport substrate or modulator that bind in the TMDs has no effect on the conformation of NBDs for crosslinking in the absence of ATP. We also determined whether the accessibility of the cysteines to FM is changed in the presence of 50 μ M CsA prior to the addition of various concentrations of FM. The IC_{50} value of FM for inhibition of ATP hydrolysis by the C431/C1074 double mutant was similar in the absence of CsA (2 μ M) and in the presence of CsA (1.4 μ M) (Figure 6).

Docking studies of FM at the ATP sites

Using the homology model of human P-gp based on Hly-B, docking studies of FM were carried out at the ATP sites with the aim to correlate biochemical data with molecular structural information. It is known that the maleimide group is reactive towards cysteine residues. Further, the X-ray structure of the FM-labeled class A β -lactamase PenP deposited in the pdb Bank (3M2J.pdb) shows the FM molecule covalently bound to a cysteine through one of the carbon atoms of the maleimide ring. Therefore, docking studies were designed to find conformations of FM, at the ATP sites and outside ATP sites, with the reactive carbon of the maleimide ring pointing towards the Walker A cysteines. Several jobs using different search boxes were carried out. Walker A lysines (K433 and K1076) that prevent modeling the FM molecule closer to the C431 and C1074, were set as flexible residues when docking FM within the ATP sites. The poses that have the maleimide ring pointing towards the Walker A cysteines with the shortest S(cys)---C(maleimide) distances were selected (Figure 7).

In ATP site 1, a FM molecule is found at the same position and orientation as ATP, sandwiched between the Walker A of NBD1 and the signature motif of NBD2. It is

interesting to note that one of the carbonyl groups of the reactive maleimide ring forms hydrogen bonds with the side-chain of neighboring residues, suggesting that these residues (E556, S434) could play a relevant role in orienting the reactive carbons towards the C431 residue. In ATP site 2, the docked FM molecule is found at a different position than the ATP although with the reactive C (maleimide) pointing towards C1074. It is difficult to know which residue/s or loops are responsible for preventing the FM molecule from docking at the same position as ATP.

Docking of FM at the ATP sites with bound ATP was also carried out. In the vicinity of ATP site 1, the FM molecule fits very well in a site defined by loops 407–409 and 602–604 and 21 the α -helix 1154–1161. FM was also docked in the equivalent site in the vicinity of ATP site 2; it is surrounded by the α -helix and loops equivalent to the ones that defined the site in the vicinity of ATP site 1 (residues 509–516, 1247–1249 and 1050–1052, respectively). At both sites, the carbonyl groups of the maleimide ring form hydrogen bonds with neighboring residues (D603 and Q1180 at site 1, and N1248 and Q535 at site 2).

Discussion

Based on the X-ray crystal structures of the mouse P-gp, Sav1866 and Hly-B, homology models of the human P-gp in both apo and closed conformations were built (Figure 1). Cysteines 431 and 1074 in the Walker A motifs of P-gp are conserved across many species. Hence, these native cysteines are excellent reporter sites to study the interaction between the two NBDs of P-gp. In this study, we selected N611C/T1256C as the other pair of double cysteine mutant P-gp (on the surface of the NBDs) located away from the ATP-binding sites. The distances in the apo and closed conformations between C431 and C1074 predicted from the human P-gp homology model are 36 Å and 32 Å respectively. With N611C and T1256C, the predicted distances in the apo and closed conformations are 56 Å and 39 Å, respectively.

With these cysteine mutations introduced in the NBDs, we first determined the expression of these mutants in insect cells as well as in the BacMam baculovirus-transduced HeLa cells.¹² As seen in Figure 2, the expression and transport function of these mutant P-gps (C431/C1074 and N611C/T1256C) are similar to those of cystless WT.

When incubated with crosslinkers M14M and M17M of spacer arm lengths 20 Å and 25 Å, respectively, disulfide crosslinking between C431 and C1074 was observed in the apo conformation but not in the ADP- V_i trapped conformation (Figure 3). Based on the homology model of the human P-gp in the apo conformation (Figure 1), the C431 and C1074 residues are exposed and freely accessible for crosslinking with the rigid crosslinkers M14M and M17M of different spacer arm lengths when P-gp is in the apo conformation. Consistent with the homology model, we showed that crosslinking is possible between the two cysteine residues, as evidenced by a crosslinked species as seen in Figure 3. Our data also showed that the NBDs of human P-gp can be closer (20 – 25 Å) than the reported distance between the NBDs in the X-ray 23 crystal structures of mouse P-gp (36 Å) and the recently reported *C. elegans* P-gp (54 Å). Considering that the distance between the C431 and C1074 residues decreases from 36 Å to 32 Å when going from the open to closed conformation, the shorter 20 – 25 Å distance observed upon crosslinking might be explained by a sliding, lateral movement of the NBDs relative to each other, different from the movement they take to form the closed conformation. This observed movement of the NBDs is consistent with the recently described highly dynamic nature of P-gp in the open conformation.²⁵

From this study, it appears that the minimal distance between NBDs appears to be 20 – 25 Å, as M8M with a spacer linker of 13 Å failed to crosslink C431 and C1074. The NBDs might be further apart (> 25 Å), but due to the unavailability of crosslinkers with arm lengths more than 25 Å, we were not able to evaluate this possibility experimentally.

To rule out the possibility that the observed crosslinked P-gp band is due to inter-molecular disulfide formation between two molecules of cysteine mutant C431/C1074, we carried out disulfide crosslinking with M17M in single C431 and C1074 mutants and in the presence and absence of 5 % β -ME (a reducing agent). No crosslinked band was observed with the single cysteine (C431 and C1074) mutants (Figure S1) and there was no difference seen in the mobility of single mutant P-gps in the presence or absence of β -ME (data not shown).

On the other hand, in the closed conformation (in the presence of ADP- V_i), there was absence of crosslinking with M14M or M17M. As there is no X-ray crystal structure of P-gp reported in the presence of bound ATP, we built a homology model of human P-gp in the closed conformation based on the crystal structure of Sav1866 (a bacterial ortholog of the human P-gp) and the Hly-B crystal structure. The model clearly shows that there is no space available for the 24 crosslinker to be accommodated at the interface of the NBDs, thus explaining the absence of crosslinking in the ADP- V_i trapped (closed) conformation.

Our results demonstrate that 1) the two native cysteines 431 and 1074 are accessible in both the apo and ADP- V_i trapped conformation to thiol-specific probes such as FM, MTS-verapamil and or MTS-rhodamine and 2) there are differences in the degree of accessibility between C431 and C1074, with C1074 having a greater accessibility to FM than C431. This observation is consistent with the report by Loo and Clarke²⁶ indicating that C1074 is more accessible to NEM (also a thiol-reactive probe) compared to C431. This difference in accessibility between the two native cysteines in the apo state and in the presence ADP- V_i suggest structural asymmetry in the NBDs, a feature of the P-gp NBDs also observed by Vigano et al.²⁷

The greater accessibility associated with C1074 compared to C431 is also evidenced by their ATPase activities (Figure 5). When 5 mM ATP was added prior to treatment with FM, the ATPase activity of C1074 was restored to a lower level of 34 %, compared to C431 with recovery of ATPase activity of 62 %. With other thiol-reactive probes, such as MTS-verapamil and MTS-rhodamine, a similar conclusion was reached supporting more accessibility of C1074 than C431. Furthermore, the inhibition of ATPase activity of the NBD cysteine mutants after reaction with probes such as MTS-verapamil (MW: 673.72) and MTS-rhodamine (MW: 695.88) also illustrated that the ATP pockets of both NBDs are accessible to molecules larger than ATP.

In the present study, we docked FM in the ATP sites to explain the accessibility of the double cysteine mutant C431/C1074 to FM in the apo and closed (ADP- V_i trapped) conformations. In the C431/C1074 double cysteine mutant, there is a similar extent of labeling with FM in both the apo and ADP- V_i trapped closed conformations. A similar extent of FM-labeling was observed in both the apo and closed conformations when the ATP pocket was saturated with ATP (in the presence of 20 mM ATP). How is it possible to access the cysteine residues at the Walker A motif when the nucleotide is bound to the NBDs? This accessibility could possibly result from the change in the orientation of the sulphur atoms of C431 and C1074 from inward-facing to outward facing with respect to the ATP pocket (Figure 7). In the absence of ATP, FM is able to occupy the ATP pocket and the maleimide moiety is directed towards the sulphur atom for covalent labeling of the cysteine. The accessibility of C1074 is slightly higher than that of C431, as reflected by the concentration of FM required to inhibit ATPase activity (IC_{50} =0.7 μ M and 1.1 μ M,

respectively). These results indicate that covalent modification of just one cysteine in the NBD is sufficient to inhibit ATPase activity, an observation consistent with that reported by Urbatsch et al.²⁸ and Loo et al.²⁶

Structural information from the X-ray crystal structures of the maltose importer²⁹ show the sulphur atoms of the Walker A cysteines in an inward orientation, facing the ATP pocket. Sav1866²² has a glycine residue instead of cysteine and Hyl-B has a serine residue, that is, however, also inward oriented. Our results suggest that the Walker A cysteines can adopt an outward orientation, at least in human P-gp, and do not form strong interactions with the ATP molecule. This interpretation is supported by modeling studies that show a FM molecule can be docked outside the ATP site with the cysteine residues adopting an outward orientation. This is consistent with reports that substitution of these cysteine residues with alanine in cysless WT have no effect on ATP-binding or hydrolysis at the NDBs or on binding of substrate at the drug binding pocket in TMDs.⁸

We also investigated the effect of disulfide crosslinking in the presence of drug substrates of P-gp. Disulfide crosslinking of cysteine residues with M17M in the NBDs is still observed with the binding of known P-gp substrates in TMDs (Figure S7), an observation also made by Loo and Clarke.³⁰ In addition, labeling with FM in the absence or presence of CsA does not change. This indicates that the binding of transport substrates in the TMDs, in the absence of ATP, does not change the accessibility of these cysteines towards covalent modification by thiol-reactive probes.

In summary, the knowledge gained through these studies concerning the accessibility of Walker A cysteines in human P-gp in both the apo and ADP-V_i trapped conformations to thiol-modifying agents is useful, as it can be applied in techniques that map conformational changes of P-gp and helps further understand its catalytic cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABC	ATP-binding cassette
P-gp	P-glycoprotein
MDR	multidrug resistance
NBDs	nucleotide-binding domains
TMDs	transmembrane domains
V_i	sodium orthovanadate
FM	fluorescein-5-maleimide
MTS	methanethiosulfonate

M8M	3,6-dioxaoctane-1,8-diyl bismethanethiosulfonate
M14M	3,6,9,12-tetraoxatetradecane-1,14-diyl bismethanethiosulfonate
M17M	3,6,9,12,15-pentaoxaheptadecane-1,17-diyl bismethanethiosulfonate
NEM	N-ethylmaleimide
CsA	cyclosporine A
DTT	dithiothreitol

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Figure 1. Homology models of human P-glycoprotein

Models of human P-gp in the apo form (open conformation) based on the X-ray structure of mouse P-gp (panel A) and in the nucleotide-bound form (closed conformation) based on an assembled model of Sav1866-TMD and Hly-B-NBD (panel B) were generated as described in Experimental Procedures. (A) Locations of cysteine mutants in NBDs in the apo conformation. The distances between cysteine pair 431 and 1074 and cysteine pair 611 and 1256 are 36 Å and 56 Å, respectively. (B) Locations of cysteine mutants in NBDs in the closed conformation. The distances between cysteine pair 431 and 1074 and cysteine pair 611 and 1256 in the closed conformations are 32 Å and 39 Å, respectively. The N-terminal and C-terminal ends of P-gp are color coded as green and cyan respectively; oxygen atom = red, nitrogen atom = blue and sulfur atom = yellow.

Figure 2. Cell surface expression and transport function of C431/C1074 and N611C/T1256C mutant P-gps expressed in HeLa cells

(A) C431/C1074 and (B) N611C/T1256C mutant P-gps have cell surface expression similar to cysless WT. HeLa cells transduced with mutant P-gp BacMam virus were evaluated for their cell surface expression by incubation with MRK-16 antibody for 1 h, followed by FITC-labeled anti-mouse secondary antibody for 30 min. (C) C431/C1074 and (D) N611C/T1256C mutant P-gps have normal transport function similar to cysless WT. The HeLa cells were incubated in the presence and absence of 1 μ M tariquidar and 0.5 mg/ml rhodamine123 (Rh123) for 45 min. Cells were washed and analyzed by flow cytometry. The histograms represent fluorescence intensity (x-axis) plotted as a function of cell number (y-axis) and are representative of three independent experiments. ³³ The figure represents a typical histogram for cell surface expression and transport function with the substrate rhodamine 123 (Rh123), for C431/C1074 (A and C, respectively) and N611C/T1256C (B and D).

Figure 3. Disulfide crosslinking of C431/C1074 with homobifunctional crosslinkers M8M, M14M and M17M in the apo and ADP-V_i trapped states and in the presence and absence of 5 mM DTT (A) Disulfide crosslinking of C431/C1074 with M8M, M14M and M17M in the apo conformation. Panel A shows the positions of the crosslinked (X-link) and the lower un-crosslinked P-gp with different crosslinkers. Membranes prepared from High-Five insect cells expressing C431/C1074 (50 μ g) were treated with 200 μ M of the disulfide crosslinkers M8M, M14M and M17M for 15 min at 4 °C. The reactions were stopped by the addition of SDS sample buffer containing no reducing agent (β -ME) and samples were subjected to immunoblot analysis on SDS/PAGE (7 % Tris-Acetate gel) as described in Experimental Procedures. (B) Disulfide crosslinking of C431/C1074 with M14M and M17M in the apo and ADP-V_i trapped states. Panel B shows the positions of the crosslinked (X-link) and

uncrosslinked P-gp with M14M and M17M in different conformational states. To generate the ADP- V_i trapped closed conformation, C431/C1074 (50 μ g) was incubated with 0.3 mM V_i , 10 mM $MgCl_2$ and 5 mM ATP and the sample was incubated for 10 mins at 37 °C, as described in Experimental Procedures. Subsequently, the sample was chilled to 4 °C for 5 mins and the crosslinkers (200 μ M) were added and incubated for 20 mins at 4 °C. (C) Disulfide crosslinking of C431/C1074 with M14M and M17M in the presence and absence of 5 mM DTT. Panel C shows the inhibition of crosslinking with M14M and M17M in the presence of 5 mM DTT. (D) Disulfide crosslinking of 34 cysless WT with crosslinkers M8M, M14M and M17M in the apo conformation. Panel D shows the absence of crosslinking of cysless WT with the various crosslinkers.

Figure 4. Labeling of cysless WT, single and double cysteine mutant P-gps with fluorescein maleimide

(A) Fluorogram (top figure) and quantification of fluorescence labeling using the software ImageQuaNT of cysless WT, C431, C1074 and C431/C1074 cysteine mutants in the apo and ADP-V_i trapped conformations. The DTT-free crude membranes of High-Five insect cells expressing cysless WT, C431, C1074 or C431/C1074 were crosslinked with 100 μ M of fluorescein maleimide for 5 min at 37 °C. To generate the ADP-V_i trapped closed conformation, single and double cysteine mutant P-gps were first incubated with 0.3 mM V_i, 20 mM MgCl₂ and 10 mM ATP and the samples were incubated for 10 mins at 37 °C prior to crosslinking with FM, as described in Experimental Procedures. The reactions were stopped by the addition of NEM (5 mM) and EDTA (50 mM) and followed by immunoprecipitation of P-gp with C219, as described in Experimental Procedures. (B)

Fluorogram (top figure) and quantification of fluorescence labeling using the software ImageQuaNT of cystless WT, N611C, T1256C and N611C/T1256C cysteine mutants in the apo and ADP- V_i trapped conformations. The DTT-free crude membranes of High-Five insect cells expressing N611C, T1256C and N611C/T1256C in both the apo and ADP- V_i trapped conformations were crosslinked with 100 μ M of FM as described in (A). The reactions were stopped by the addition of NEM (5 mM) and EDTA (50 mM) and followed by immunoprecipitation of P-gp with C219 as described in Experimental Procedures. The same gel is stained with colloidal blue dye for detection of P-gp expression (bottom figure), as described in Experimental Procedures. The average values from two independent experiments for ADP- V_i trapped condition are shown. (C) Fluorogram and quantification of fluorescence labeling using the software ImageQuaNT of cystless WT and double C431/C1074 cysteine mutants in the apo conformation, in the presence of saturating ATP (20 mM) and $MgCl_2$ (20 mM) and ADP- V_i trapped conformations. The DTT-free cystless WT, C431/C1074 in the apo state, in the presence of saturating ATP (20 mM) and $MgCl_2$ (20 mM) and in the ADP- V_i trapped conformations was crosslinked with 100 μ M of FM. In this experiment, 0.3 mM V_i , 20 mM $MgCl_2$ and 20 mM ATP were added to generate the ADP- V_i conformation and the samples were incubated for 10 mins at 37 °C prior to treatment with FM, as described in Experimental Procedures. The gels in panels A, B and C were stained with colloidal blue dye for detection of P-gp expression as described in Experimental Procedures, fixed and air-dried in DryEase mini cellophane (Novex). The scans of stained air-dried gels are shown under the respective fluorogram in panels A, B and C. The quantified data are shown in the histogram and represent the average of at least two experiments. Data with error bars denote SD ($n = 3$).

Figure 5. Effect of fluorescein maleimide on P-gp-mediated ATP hydrolysis by cystless WT, single C431, C1074 and double C431/C1074 mutant P-gps

(A) Inhibition of P-gp ATPase activity by FM in cystless WT (■), C431 (▲), C1074 (▼) and C431/C1074 (◆). Vanadate-sensitive P-gp-mediated ATPase hydrolysis was measured as described in Experimental Procedures in the presence of increasing concentrations of FM (0 – 25 μM). FM was incubated with the respective cysteine mutants (10 – 20 μg protein/100 μL) for 5 mins at room temperature prior to the addition of 5 mM ATP. The IC₅₀ (FM) for inhibition of P-gp mediated ATP hydrolysis in cystless WT cannot be determined, as FM does not interact with cystless P-gp. The IC₅₀ (FM) values for C431, C1074 and C431/C1074 are 1.1 (0.15) μM, 0.70 (0.1) μM and 0.63 μM, respectively. (B) Inhibition of P-gp ATPase activity by FM after pre-36 incubation with 5 mM ATP in C431, C1074 and C431/C1074. 5 mM of ATP and 20 mM MgCl₂ is added to the reaction mixture on ice for 5 mins with the respective cysteine mutants (10 – 20 μg protein/100 μL) prior to the addition of various concentrations of FM. The IC₅₀ (FM) for inhibition of P-gp-mediated ATP hydrolysis in cystless WT, C431, C1074 and C431/C1074 cannot be determined when 5 mM ATP is added prior to FM due to partial inhibition. (C) Irreversibility of ATPase activity after addition of 4 mM DTT. In this experiment, 5 μg of C431/C1074 crosslinked with 100 μM FM in both the apo and closed conformations was evaluated for ATPase activity with and without 4 mM DTT. Data represent the mean values (Error bars showing mean ± SD are shown for n = 3). In panels A and B, IC₅₀ curves were generated by non-linear regression (one phase decay) using GraphPad Prism 5.

Figure 6. Effect of cyclosporine A on double C431/C1074 mutant P-gp-mediated ATP hydrolysis in the presence of increasing concentrations of FM

Vanadate-sensitive ATPase hydrolysis by the double cysteine mutant was measured as described in Experimental Procedures in the presence of increasing concentrations of FM (0 – 5 μ M) after pre-incubation with no substrate (●) and 50 μ M CsA (■). In this experiment, 50 μ M CsA was incubated with C431/C1074 (20 μ g protein/100 μ L) for 5 mins at room temperature prior to the addition of 5 mM ATP. The IC_{50} (FM) for inhibition of P-gp mediated ATP hydrolysis in the absence and the presence of CsA is 2.0 ± 0.2 μ M and 1.4 μ M, respectively. Data represent the mean values (error bars showing $\pm$ SD are shown for $n = 3$). IC_{50} curves were generated by non-linear regression (one phase decay) using GraphPad Prism 5.

Figure 7. Modeling of orientations of conserved C431 in NBD1 and C1074 in NBD2 in human P-gp

The homology model of NBDs of human P-gp using the HlyB NBD structure was generated as described in Experimental Procedures. (A) Modeling of FM at the NBD1 in the absence of ATP. The FM molecule is docked at the NBD1 using the program Vina-Autodock, with the maleimide moiety directed towards C431 for thiol modification. K-433 is made flexible for the docking of FM in the NBD1. (B) Modeling of FM at the NBD1 in the presence of ATP. C431 is oriented outwards for chemical reaction with the maleimide moiety of FM. (C) Modeling of FM at the NBD2 in the absence of ATP. The FM molecule is docked at the NBD2 using the program Vina-Autodock, with the maleimide moiety directed towards C1074 for thiol modification. (D) Modeling of FM at the NBD2 in the presence of ATP. C1074 is oriented outwards for chemical reaction with the maleimide moiety of FM. Selected residues are shown in stick models and labeled. The NBD model is shown in two orientations: (A and B) NBD1 to the left and NBD2 to the right; (C and D) NBD1 to the right and NBD2 to the left. The figure is colored as follows: NBD1 = green, NBD2 = cyan; ATP = black; FM = magenta; C431 and C1074 = red; and created with PyMOL (The PyMOL Molecular Graphics System, Schrodinger, LLC).