Evidence for Simultaneous Binding of Dissimilar Substrates by the *Escherichia coli* Multidrug Transporter MdfA†

Oded Lewinson and Eitan Bibi*

Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT: The mechanism by which multidrug transporters interact with structurally unrelated substrates remains enigmatic. Based on transport competition experiments, photoaffinity labeling, and effects on enzymatic activities, it was proposed in the past that multidrug transporters can interact simultaneously with a number of dissimilar substrate molecules. To study this phenomenon, we applied a direct binding approach and transport assays using the *Escherichia coli* multidrug transporter MdfA, which exports both positively charged (e.g., tetraphenylphosphonium, TPP^+), zwitterionic (e.g., ciprofloxacin), and neutral (e.g., chloramphenicol) drugs. The interaction of MdfA with various substrates was examined by direct binding assays with the purified transporter. The immobilized MdfA binds TPP^+ in a specific manner, and all the tested positively charged substrates inhibit TPP^+ binding. Surprisingly, although TPP^+ binding is not affected by zwitterionic substrates, the neutral substrate chloramphenicol stimulates TPP^+ binding by enhancing its affinity to MdfA. In contrast, transport competition assays show inhibition of TPP^+ transport by chloramphenicol. We suggest that MdfA binds TPP^+ and chloramphenicol simultaneously to distinct but interacting binding sites, and the interaction between these two substrates during transport is discussed.

Eukaryotic and prokaryotic cells often become multidrug resistant due to an elevated levels of expression of multidrug transporters (Mdrs),[1] which recognize and consequently remove many chemically unrelated toxic compounds from the cell cytoplasm or cytoplasmic membrane to the external medium. In bacteria, a large variety of distinct Mdrs have been identified (*1*−*7*), and it is now clear that pathogenic bacteria also have multidrug resistance mechanisms that pose a serious potential clinical threat (*8*). Similar to P-glycoprotein-mediated multidrug resistance in mammalian systems (*9*), prokaryotic Mdr transporters are also able to extrude a variety of structurally unrelated lipophilic compounds, many of which are positively charged under physiological conditions. However, there are bacterial Mdr proteins that also interact with neutral and zwitterionic drugs, some of which are relatively hydrophilic, and some transporters export lipophilic anionic drugs (*7*, *10*−*14*). Despite intensive efforts to understand the transport-related multidrug resistance phenomenon, substrate recognition by Mdrs remains unresolved and probably differs substantially from that of specific transporters such as LacY (*15*, *16*). Therefore, in addition to potential clinical importance, Mdr proteins pose intriguing questions regarding substrate recognition and transport mechanism.

The general question of how secondary Mdr transporters actually recognize dissimilar drugs has been addressed recently by analyzing the kinetics of transport-competition experiments. Briefly, competitive, noncompetitive, or uncompetitive inhibition was observed with various substrates of the MFS-related Mdr transporters QacA (*17*) and LmrP (*18*). Based on these results, it was suggested that both Mdrs possess at least two distinct drug interaction sites. Similar conclusions were drawn from experiments with photoactivatable substrates of ABC-related Mdr transporters (*19*−*23*). Our recent studies (*24*) and those described here focus mainly on substrate specificity determinants and the drug recognition properties of the *E. coli* multidrug resistance transporter MdfA [also termed Cmr; (*25*)], which represents multidrug transporters that recognize positively charged, zwitterionic, and uncharged substrates (*12*, *26*). MdfA is a 410 amino acid residue MFS-related membrane protein. Cells expressing MdfA from a multicopy plasmid exhibit multidrug resistance due to active drug extrusion driven by the proton electrochemical gradient (interior negative and alkaline). Recent studies (*26*) have proposed that MdfA is a drug/proton antiporter. In this study, the multidrug recognition phenomenon was addressed by direct substrate binding assays. For this purpose, MdfA was purified, and immobilized essentially as described by Muth and Schuldiner (*27*). The results demonstrate that MdfA binds TPP^+ in a specific manner and that in the presence of chloramphenicol MdfA binds both substrates (TPP^+ and chloramphenicol) simultaneously.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]TPP^+ (32 Ci/mmol) was purchased from Amersham and [^{3}H]chloramphenicol from NEN. EtBr, chloramphenicol, kanamycin, mitomycin, Hoechst 33342,

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* To whom correspondence should be addressed. Fax: 972-8-9344118; Phone: 972-8-9343464; E-mail: bcbibi@wicc.weizmann.ac.il.

[1] Abbreviations: TPP^+, tetraphenylphosphonium; Mdr, multidrug transporter; DDM, *N*-dodecyl-maltoside; LB, Luria−Bertani medium; NTA, nitrilotriacetic acid; EtBr, ethidium bromide.

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DNase, and ampicillin were all purchased from Sigma, and the protease inhibitor Pefablock was from Boehringer Mannheim. Restriction and modifying enzymes were obtained from New England BioLabs. DDM was purchased from Anatrace. Prestained protein molecular weight markers were obtained from New England BioLabs, and DNA molecular weight markers were from Fermentas. GeneClean glassmilk DNA purification kits were obtained from Bio 101, and Wizard plasmid prep kits were from Promega. India HisProbe (Pierce) was used for MdfA-6His detection by Western blotting. His Bind resin was obtained from Novagen and used for purification of MdfA-6His. For binding assays, His Bind resin from Qiagen was utilized. All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. The construction of plasmid pUC18/pARA/MdfA6His was performed as follows: Plasmids pT7-5/pARA/MdfA-BAD and pT7-5/pTaq/MdfA6His (Adler, J., and Bibi, E., unpublished data) were digested with the restriction endonuclease *Aat*II, and the short MdfA6His fragment was ligated with the long fragment released from pT7-5/pARA/MdfA-BAD. The resulting plasmid, pT7-5/pARA/MdfA6His, was then cleaved by restriction endonucleases *Hin*dIII and *Sac*I, and the DNA fragment harboring the arabinose promoter and the MdfA6His encoding gene (2643 bp) was ligated with plasmid pUC18, which was digested with the same restriction enzymes. The product of this ligation, plasmid pUC18/pARA/MdfA6His, was used for overexpression of MdfA. The outer membrane permeability mutant *E. coli* UTL2*mdfA*::*kan* (*28*, *24*) was transformed with this plasmid and used for overexpression of MdfA.

Growth Conditions. E. coli UTL2*mdfA*::*kan*(pUC18/pARA/MdfA6His) cultures were grown at 37 °C in LB supplemented with ampicillin (100 μg/μL), and kanamycin (30 μg/μL). Overnight cultures were diluted to 0.07 OD_{600} unit, grown up to $OD_{600} = 1$, and induced with 0.2% arabinose for 1.5 h. A typical 12 L culture yielded 17 g (wet weight) of cells.

Preparation of Membranes. For the removal of outer membranes, cell pellets were washed once in 0.1 M Tris-HCl buffer (pH 7.9) containing 1 mM EDTA, and resuspended in the same buffer containing 0.25 M sucrose and 50 μg/mL lysozyme per 1 g of cells (wet weight) in 100 mL. After 15 min of incubation at room temperature, $MgSO_4$ was added to a final concentration of 10 mM. The cells were then incubated for another 20 min at 4 °C and pelleted by centrifugation (8000*g*, 1 h). The resulting spheroplasts were resuspended (1 g in 5 mL) in KPi buffer (pH 7.5) containing 5 mM $MgSO_4$, 1 mM β-mercaptoethanol, 100 μg/mL DNase, and 0.5 mM Pefablock, and passed 4 times through a French pressure cell (20 000 psi). Sucrose and NaCl were then added to final concentrations of 0.25 and 0.5 M, respectively, and membranes were collected by ultracentrifugation (2 h, 250000*g*). Finally, the pellets were resuspended and homogenized in buffer A [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 5 mM imidazole, and 5% glycerol] and then frozen in liquid nitrogen.

Membrane Solubilization and MdfA Purification. For solubilization, membranes were thawed at room temperature, and DDM was added to 1.2%. The mixtures were then agitated gently for 30 min at 4 °C. Insoluble material was discarded by ultracentrifugation (250000*g*, 1 h), and the soluble fraction was loaded onto a Ni−NTA column. Unbound material was washed with buffer A containing 40 mM imidazole, and the bound MdfA-6His was eluted with buffer A containing 250 mM imidazole. The purification procedure was conducted using a peristaltic pump, at a flow rate of 150 mL/h. The purity of the fractions was evaluated by silver and Coomassie staining of SDS−PAGE gels. Protein concentration in the purified fractions was estimated by the method of Peterson (*29*). For dialyzed fractions in which the imidazole was diluted to 0.5 mM, the protein was estimated according to Lowry et al. (*30*) and by the absorbance at 280 nm. Purified MdfA was immediately subjected to binding assays as described in the following section.

Binding Assays. The binding assays were based on a method developed by Muth and Schuldiner (*27*), with the following modifications: All binding assays were conducted at 4 °C in 20 mM Tris-HCl buffer (pH 7) containing 0.5 M NaCl and 0.1% DDM (buffer B). Initially, the purified MdfA-6His was incubated with the His Bind resin (25 min at 4 °C) (using a MdfA-6His:resin ratio of 1:1 μg/μL); the unbound material was discarded by pulse centrifugation. Next, MdfA-6His bound to resin was resuspended in 200 μL of buffer B, and incubated (10 min) with the indicated concentration of the radiolabeled test substrate. In competition experiments, the unlabeled competitor is added together with the labeled test substrate. After incubation with the labeled substrate, an aliquot of 180 μL of the resin mixture was transferred to a Promega Wizard minicolumn on top of an eppendorf tube (1.5 mL) and centrifuged at 10000*g* for 20 s. Unbound (flowthrough) material was discarded, and MdfA-6His−resin was resuspended in 100 μL of buffer B containing 350 mM imidazole. The radioactivity of this suspension was measured using liquid scintillation. The results in Figures 2B, 4A, and 4B are represented as nonlinear regression curves generated by the computer program Kaleidagraph 3.0.4 and used for calculation of K_I and K_S values. All K_D values were calculated from Scatchard plots.

Transport Assays. For TPP^+ transport, overnight cultures of *E. coli* UTL cells were diluted to 0.05 OD_{600} unit and grown at 37 °C in LB supplemented with ampicillin (100 μg/μL) and kanamycin (30 μg/μL) to 0.3 OD_{600} unit. Cells were then collected and washed once in an equal volume of ice-cold 50 mM potassium phosphate buffer, pH 7.3, and resuspended in the same buffer to 13 OD_{420} units. Following a 4 min recovery at 33 °C in the presence of 20 mM lithium lactate, and chloramphenicol where indicated, transport was initiated by addition of the desired tritiated TPP^+ concentration. Transport was terminated by rapid filtration as previously described (*12*). Chloramphenicol transport was conducted in a similar manner (*12*). Cultures were grown to 0.5 OD_{600} unit, washed in 50 mM potassium phosphate buffer, pH 7, and resuspended to 8 OD_{420} units. Following a 20 min recovery at 37 °C in the presence of 0.2% glucose, transport was initiated by addition of the desired radiolabeled chloramphenicol concentration and TPP^+ where indicated.

Western Blotting. Membrane fractions, solubilized fractions, and purified MdfA-6His fractions were subjected to 12% SDS−PAGE. Proteins were electroblotted to nitrocellulose membranes, and after incubation with the 6His-specific India HisProbe solution, the membranes were probed by ECL.

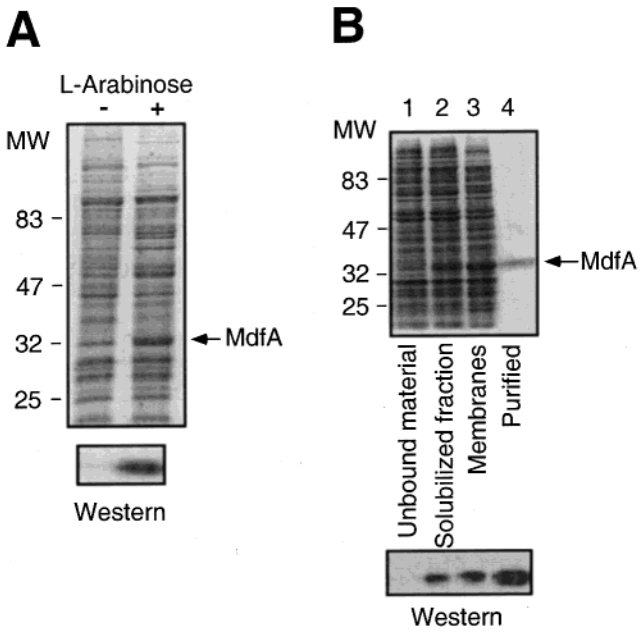

FIGURE 1: Overexpression and purification of MdfA. (A) Overexpression of MdfA. Cells harboring pUC18 with or without the *mdfA* gene (encoding 6His-tagged proteins) were induced at $OD_{600} = 1$ with arabinose (0.2%) for 1.5 h, and harvested. Membranes were prepared as described under Experimental Procedures and collected by ultracentrifugation. Samples (25 μg of proteins) were separated by SDS−PAGE and stained with Coomassie blue. (B) Purification of MdfA. DDM-solubilized membranes were purified on a nickel−NTA column, and samples from the indicated fractions (25 μg of protein, lanes 1−3; 8 μg of protein, lane 4) were separated by SDS−PAGE and stained with Coomassie blue. The 6His-tagged proteins (1 μg samples) were subjected to Western blot analysis (A and B, lower panels) as described under Experimental Procedures.

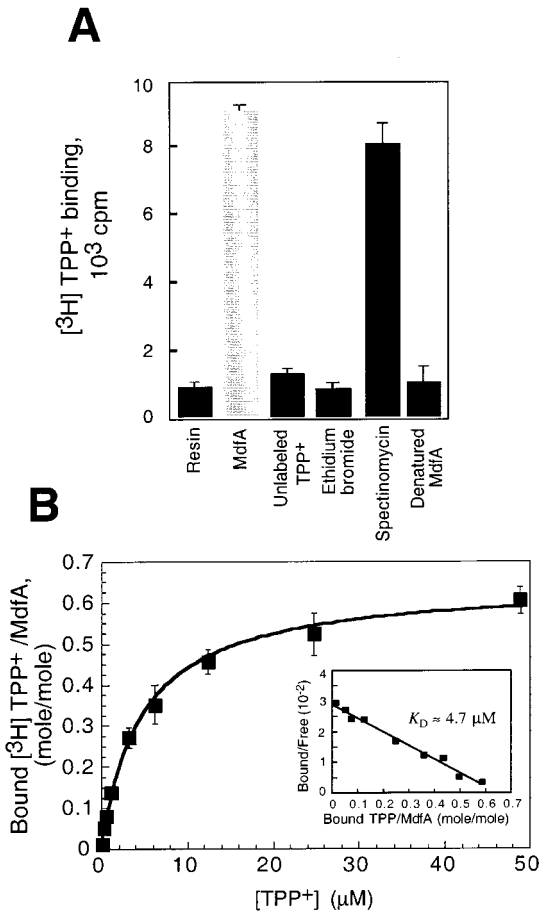

FIGURE 2: Specific binding of TPP^+ by MdfA-6His. (A) Purified MdfA-6His or denatured protein (5 min boiling in 1% SDS) in buffer A or in buffer A without MdfA was incubated with Ni−NTA resin, and the supernatant was discarded after brief centrifugation. The pelleted beads were resuspended in buffer A containing 1 μM [^{3}H]TPP^+ and the indicated additions (1 mM unlabeled TPP^+; 250 μM EtBr; 250 μM spectinomycin). Radioactivity retained by the beads was assayed as described under Experimental Procedures. (B) Binding of increasing concentrations of [^{3}H]TPP^+ to MdfA was measured as described. A K_D of ~4.7 μM for TPP^+ was derived from the Scatchard plot (inset). The nonspecific component of binding to resin alone was subtracted. The experiments were performed as triplicates and repeated 3 times. Error bars are indicated for errors greater than 1% of the total counts.

RESULTS

Overexpression, Solubilization, and Purification of MdfA. As discussed previously (*25*), the *mdfA* gene does not contain a classical promoter, and the mechanism by which expression is regulated is unknown. Attempts to overexpress the protein with *mdfA*, under the control of various promoters (*31*), did not yield expression levels sufficient for biochemical studies. To improve expression, a gene encoding MdfA with a 6His tag at the C-terminus was transferred to the high copy-number plasmid pUC18 under control of the tight *araB* promoter. In this manner, the toxic effect of overexpression is alleviated, and after induction with arabinose, high expression levels of MdfA-6His are achieved (Figure 1A). Tight control of the *araB* promoter enables the cells to grow to the desired cell density for induction after which complete arrest of growth is observed (data not shown). Membranes from cells overexpressing MdfA-6His were solubilized with DDM, and the protein was purified by Ni−NTA chromatography. A single step yields nearly homogeneous MdfA-6His (Figure 1B).

Specific Binding of TPP^+ by Immobilized MdfA. Purified MdfA-6His was immobilized by incubation with Ni−NTA agarose, and the unbound material was discarded by brief centrifugation. The pellet was resuspended in buffer containing 1 μM [^{3}H]TPP^+, and after incubation, the beads were collected by filtration and assayed for bound ligand by scintillation spectrometry. As a control, radioactivity retained by Ni−NTA agarose without bound protein that was treated in an identical fashion was measured. Binding of TPP^+ to resin with bound MdfA is 10-fold higher than that observed with protein-free resin (Figure 2A). Maximal TPP^+ binding is achieved after 10 min of incubation (data not shown). To examine whether the observed binding is specific, several additional experiments were performed. Inactivation of MdfA by boiling in 1% SDS for 5 min prior to immobilization abolishes the specific components of TPP^+ binding (Figure 2A). Upon addition of excess unlabeled substrate, [^{3}H]TPP^+ binding was lost (Figure 2A). Moreover, incubation of the immobilized MdfA with the positively charged MdfA substrate EtBr completely blocks TPP^+ binding, whereas spectinomycin, which is not a MdfA substrate (*12*), or lactose (data not shown) does not affect TPP^+ binding (Figure 2A). As expected from its specific nature, binding of TPP^+ to MdfA is saturable (Figure 2B). Equilibrium binding of TPP^+ was measured over a broad range of concentrations, and as shown (Figure 2B, inset), MdfA binds TPP^+ with a K_D of

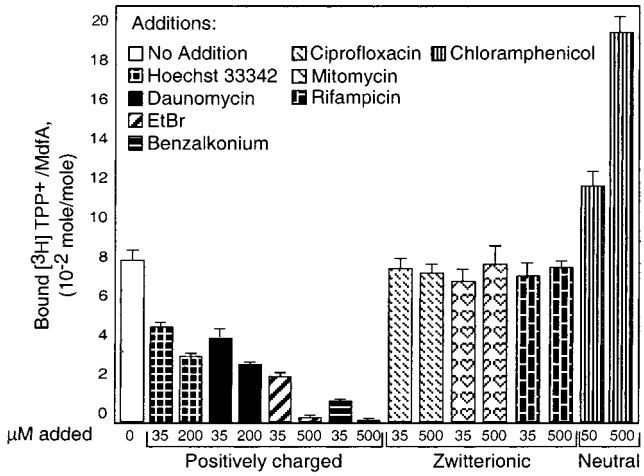

FIGURE 3: Effect of differently charged substrates on TPP^+ binding to MdfA. Binding of [^{3}H]TPP^+ (1 μM) in the absence or presence of the indicated substrates was performed as described in Figure 2. The nonspecific component of binding to resin alone was subtracted. The experiments were performed as triplicates and repeated 3 times. Error bars are indicated for errors greater than 1% of the total counts.

~4.7 μM. The calculated stoichiometry of bound $[TPP]^+$ to [MdfA] suggests that a fraction (35%) of the immobilized MdfA molecules is not functional. Although at this stage the possibility that MdfA is functional as an oligomer cannot be ruled out, the calculated stoichiometry does not support this notion.

Effect of Differently Charged Drugs on [^{3}H]TPP^+ Binding by MdfA. As shown above (Figure 2A), the positively charged substrate EtBr blocks TPP^+ binding to MdfA. To test the effect of other positively charged, zwitterionic, and neutral substrates on TPP^+ binding, immobilized MdfA was incubated with radiolabeled TPP^+ alone or in the presence of various concentrations of either of the indicated substrates (Figure 3). As shown, all the cationic substrates (EtBr, daunomycin, benzalkonium, and Hoechst 33342) inhibit TPP^+ binding to MdfA, whereas the zwitterionic compounds (ciprofloxacin, rifampicin, and mitomycin) have no effect. In contrast, in the presence of chloramphenicol, TPP^+ binding by MdfA is increased significantly. Clearly, such a stimulatory effect indicates simultaneous interaction of both substrates with the protein. Several control experiments were performed in order to test the validity of this hypothesis (data not shown): (i) The possibility that chloramphenicol may influence the absorption of TPP^+ to the Ni−NTA resin was ruled out by testing simultaneous binding to protein-free resin. (ii) We examined the possibility that chloramphenicol may stabilize MdfA, either by protection against degradation during the experiment or by inducing a stable, active conformation. Stability of the immobilized MdfA was tested over time courses equivalent to or longer than those used for the binding assays. The results demonstrate stable binding of TPP^+ to MdfA for up to 1.5 h, under the same conditions used for the binding assays. (iii) The possibility that the effect of chloramphenicol on TPP^+ binding to MdfA is due to stabilization of a specific conformer of MdfA by chloramphenicol was ruled out by experiments in which immobilized MdfA was incubated first with chloramphenicol, washed, and exposed immediately to the labeled TPP^+. Since no effect of chloramphenicol on TPP^+ binding is observed under these conditions, stimulation of TPP^+ binding requires the presence of chloramphenicol during the assay.

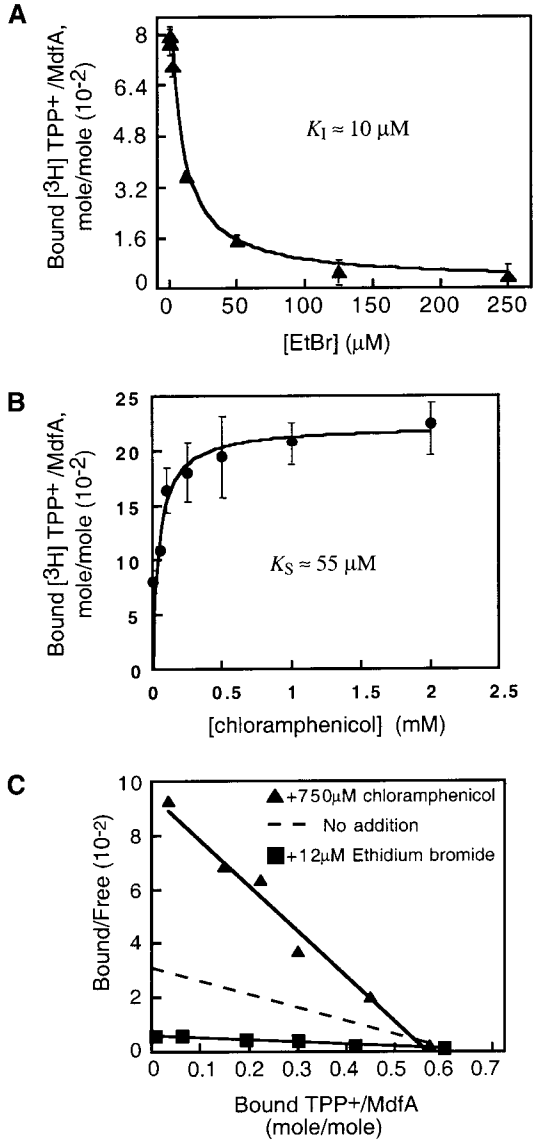

FIGURE 4: Effects of EtBr and chloramphenicol on TPP^+ binding to MdfA. (A) Binding of [^{3}H]TPP^+ (1 μM) to immobilized MdfA was measured in the presence of increasing concentrations of EtBr (0.5−250 μM). (B) Binding of [^{3}H]TPP^+ (1 μM) to immobilized MdfA was measured in the presence of increasing concentrations of chloramphenicol (50−2000 μM). (C) Binding of increasing [^{3}H]-TPP^+ concentrations to MdfA was measured in the presence of chloramphenicol (750 μM) or EtBr (20 μM). K_D values were calculated from the Scatchard plots as presented. The calculated K_D for TPP^+ binding (~4.7 μM, without addition; see Figure 2) was changed to ~32 μM in the presence of EtBr and to ~1.7 μM in the presence of chloramphenicol. The nonspecific component of binding to resin alone was subtracted in all cases. The experiments were performed as triplicates and repeated 3 times. Error bars are indicated for errors greater than 1% of the total counts.

Further characterization of the opposite effects of EtBr and chloramphenicol on TPP^+ binding to MdfA was accomplished by measuring their inhibitory and stimulatory constants, respectively. Experiments with EtBr were conducted at various concentrations of TPP^+, and the results show that EtBr inhibits TPP^+ binding to MdfA with a K_I of 10 μM, suggesting that MdfA binds EtBr or TPP^+ with affinities of the same order of magnitude. A representative

experiment with 1 μM TPP^+ is shown in Figure 4A. Equilibrium binding of increasing TPP^+ concentrations in the presence of EtBr demonstrates that EtBr inhibits TPP^+ binding to MdfA in a competitive fashion and that the maximal binding at saturating TPP^+ concentrations is not affected by EtBr (Figure 4C). In contrast to the inhibitory effect of EtBr, chloramphenicol stimulates TPP^+ binding to MdfA with a K_S of approximately 55 μM (Figure 4B), indicating that MdfA binds chloramphenicol with a lower affinity than EtBr or TPP^+. Unfortunately, due to its low affinity, we were so far unable to measure directly the K_D for chloramphenicol binding to MdfA. Importantly, even at high chloramphenicol concentrations, no decrease in TPP^+ binding is observed, suggesting that TPP^+ and chloramphenicol do not bind to a common binding site (Figure 4B).

The stimulatory effect of chloramphenicol on TPP^+ binding to MdfA can be explained by either of two possibilities: (i) one substrate may increase the number of binding sites available to the other substrate; or (ii) binding of the first substrate may increase the affinity of the second substrate. To distinguish between these two possibilities, equilibrium binding of increasing TPP^+ concentrations to MdfA was measured in the presence or absence of various chloramphenicol concentrations. The results indicate that the affinity of the binary chloramphenicol−MdfA complex for TPP^+ is increased. Thus, in the presence of 750 μM chloramphenicol, the K_D for TPP^+ decreases from ~4.7 to ~1.7 μM (Figure 4C). Importantly, the maximal binding at saturating TPP^+ concentrations is not affected by chloramphenicol. Therefore, the stimulatory effect of chloramphenicol on TPP^+ binding is due to an increase in affinity and not to an increase in the number of binding sites.

Effect of TPP^+ and Chloramphenicol on Each Other's Transport by Cells Expressing MdfA. To determine in what way the binding−stimulatory effect influences the catalytic transport cycle, we examined transport of one substrate in the absence or presence of the other substrate. As shown in Figure 5, transport of TPP^+ (0.2 mM) is inhibited by increasing chloramphenicol concentrations, and complete inhibition is achieved at 0.8 mM chloramphenicol (panel A). Correspondingly, transport of chloramphenicol (2 μM) is inhibited by increasing TPP^+ concentrations, with complete inhibition at 80 μM TPP^+ (panel B). These results show that the binding−stimulatory effect is translated into a transport-inhibitory effect, and this phenomenon is discussed below.

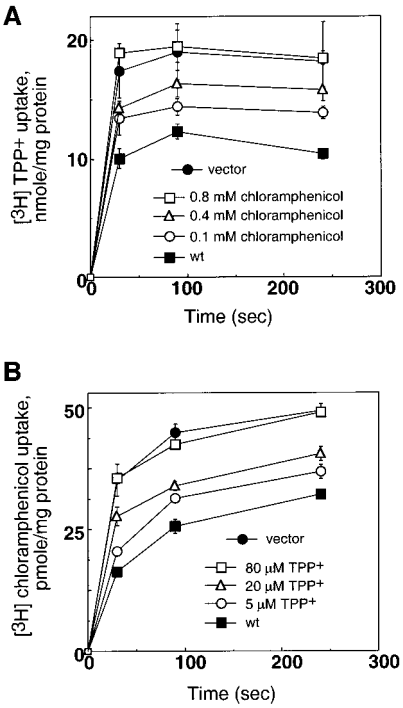

FIGURE 5: Transport competition experiments with TPP^+ and chloramphenicol. (A) Uptake of [^{3}H]TPP^+ (0.2 mM) was measured in *E. coli* UTL cells harboring vector alone or MdfA-encoded plasmid (wt) in the absence or presence of various chloramphenicol concentrations, as indicated. (B) Similarly, uptake of [^{3}H]chloramphenicol (2 μM) was measured in the absence or presence of various TPP^+ concentrations, as indicated. Error bars are indicated for errors greater than 1% of the total counts.

DISCUSSION

The ability of Mdr transporters to confer resistance against an extremely broad spectrum of toxic agents has led to interesting mechanistic hypotheses. The prevailing hypothesis favors a direct mechanism by which Mdr transporters can recognize a variety of compounds and actively export them across or out of the membrane. Such a mechanism implies that a typical Mdr transporter must be able to recognize and interact with a variety of dissimilar compounds. This property of Mdr transporters has been investigated recently using several experimental methods, and it was suggested that Mdr transporters exhibit at least two substrate-interaction sites. Putman et al. (*18*) concluded from the kinetics of transport-competition experiments performed with the MFS-related Mdr transporter LmrP that the antiporter has at least two drug-interaction sites, which may represent distinct sites or a common, bifunctional binding site. Two distinct drug binding sites, for mono- and divalent cationic substrates, have also been proposed for another MFS-related Mdr transporter, QacA, as a result of studying the interactions between various substrates and QacA by using transport-competition assays (*17*). Use of photoaffinity labeling and drug-stimulated ATPase activity also suggests that ABC-related Mdr transporters possess multiple drug binding sites that can be occupied simultaneously (*19*−*23*). Thus, based on various experimental approaches, the current working model is that Mdr transporters may interact simultaneously with at least two dissimilar substrates or modulators. Here, for the first time, we studied this phenomenon by direct binding assays with the *E. coli* multidrug transporter MdfA, and the results strongly support this view.

Initially and most importantly, we show that binding of TPP^+ to MdfA is specific. First, binding of the labeled substrate is decreased by excess unlabeled substrate, suggesting substrate specificity. Second, denaturation of MdfA prior to incubation with the substrate completely abolishes binding. Furthermore, binding of TPP^+ is satiable, indicating a limited number of specific binding sites. Calculation of the binding stoichiometry for TPP^+ to MdfA yields a molar ratio of about 0.65, suggesting that a fraction of the immobilized transporter is inactive. The substrate binding saturation curve and the linearity of the Scatchard plot suggest a single binding site for TPP^+.

Previously, it was observed that mutations at position 26 (E26) in MdfA affect drug resistance to a variety of toxic compounds including chloramphenicol, EtBr, and TPP^+ (*24*;

Adler, J., and Bibi, E., unpublished data). We speculated that E26 is involved in overlapping but distinct binding sites for the different substrates or plays an important role in a common, versatile binding site. Similar conclusions may be drawn from the observations that various substrates inhibit the MdfA-mediated efflux of EtBr (*12*, *24*, *26*). The ability of MdfA to bind TPP^+ with a measurable affinity (K_D of $\sim$4.7 μM) provided the means for studying the interaction of the purified transporter with the different substrates also by binding assays. It is demonstrated that TPP^+ binding is inhibited by EtBr, benzalkonium daunomycin, and Hoechst 33342, all of which are positively charged MdfA substrates, suggesting that these substrates compete for binding to the same site. Further characterization of the effect of EtBr on TPP^+ binding established the competitive nature of this inhibition (Figure 4). Interestingly, TPP^+ binding is unaffected by all tested zwitterionic substrates, suggesting that these drugs do not compete with TPP^+ for binding to the same site in MdfA (Figure 3). Surprisingly, in contrast to these two groups of substrates, the neutral substrate chloramphenicol markedly stimulates TPP^+ binding to MdfA. Importantly, stimulation results from an increase in affinity and not from a change in the number of binding sites. These studies demonstrate that MdfA binds chloramphenicol and TPP^+ simultaneously, thereby lending strong support to the suggestion that MdfA and possibly other Mdr transporters possess a few distinct substrate binding sites. The cooperativity in binding of chloramphenicol and TPP^+ to MdfA indicates that the two binding sites must communicate with each other. We propose that chloramphenicol and TPP^+ either bind to different domains of a common hydrophobic pocket in MdfA, or bind to separate sites that interact allosterically with one another. In summary, we suggest that the various interactions between the different MdfA substrates mainly reflect their electrical properties and not their sizes or chemical structures.

Attempts to correlate MdfA-mediated efflux and binding are speculative since no assignment of directionality can be made with substrate binding to the immobilized transporter. In any case, the following relationships between transport and binding are noteworthy. Wild-type MdfA confers significantly higher levels of resistance to chloramphenicol than to TPP^+. Similarly, when compared to control cells, the transport affinity of chloramphenicol seems much higher than that of TPP^+ (*12*; unpublished data). In contrast, the binding affinity of MdfA for TPP^+ is significantly higher than that of chloramphenicol. Similar observations have been made with the SMR-related Mdr transporter EmrE (*27*). TPP^+, a potent inhibitor of EmrE, binds the transporter with an extremely high affinity. By comparison, only modest inhibition of TPP^+ binding is observed in the presence of extremely high concentrations of methyl viologen, although EmrE-mediated resistance and transport of methyl viologen are significantly higher than those observed with TPP^+. Therefore, it is tempting to assume that the binding affinity of a specific substrate is inversely related to its transport rate. Although our transport competition experiments with MdfA support this notion, the findings from these two systems (EmrE and MdfA) are only at a preliminary stage and include few substrates. Future work is needed to determine if such an inverse relation actually exists.

Finally, it would be interesting to investigate the interaction between chloramphenicol and TPP^+ and other substrates during the transport cycle in a reconstituted system. Is there a common transport pathway for different substrates, or are they translocated independently through distinct transport pathways? In this regard, recent studies with AtMRP2 suggested that this ABC transporter simultaneously translocates dissimilar substrates (*32*). Such a consideration may have important clinical implications that should be considered when treating Mdr cells with a cocktail of drugs if all are substrates for the same Mdr transporter. The intriguing relationship between a positively charged substrate and a neutral substrate presented here also raises interesting possibilities concerning the design of such cocktails.

In the future, we plan to use this powerful binding assay in order to investigate further the multidrug binding properties of various MdfA mutants and the interactions between other pairs of substrates and MdfA. Preliminary results suggest that the stimulatory relationship between chloramphenicol and TPP^+ may not be limited to TPP^+. However, attempts to identify additional neutral substrates for MdfA have been unsuccessful so far. In addition, it might be possible to map the substrate binding pocket of MdfA by a combination of genetic studies and binding experiments (*15*), both with MdfA-reconstituted liposomes and with immobilized MdfA. This information will undoubtedly increase our understanding of the mechanism of transport-mediated multidrug resistance phenomena in general.

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