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The multidrug transporter MdfA deviates from the canonical model of alternating access of MFS Transporters

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Abstract

The prototypic multidrug (Mdr) transporter MdfA from *E. coli* efflux chemically-dissimilar substrates in exchange for protons. Similar to other transporters, MdfA purportedly functions by alternating access of a central substrate binding pocket to either side of the membrane. Accordingly, MdfA should open at the cytoplasmic side and/or laterally toward the membrane to enable access of drugs into its pocket. At the end of the cycle, the periplasmic side is expected to open to release drugs. Two distinct conformations of MdfA have been captured by X-ray crystallography: An outward open (O_o) conformation, stabilized by a Fab fragment, and a ligand-bound inward-facing (I_f) conformation, possibly stabilized by a mutation (Q131R). Here, we investigated how these structures relate to ligand-dependent conformational dynamics of MdfA in lipid bilayers. For this purpose, we combined distance measurements by Double Electron Electron Resonance (DEER) between pairs of spin labels in MdfA reconstituted in nanodiscs with cysteine cross-linking of natively expressed membrane-embedded MdfA variants. Our results suggest that in a membrane environment, MdfA assumes a relatively flexible, outward-closed/inward-closed (O_c/I_c) conformation. Unexpectedly, our data show that neither the substrate TPP nor protonation induces large scale conformational changes. Rather, we identified a substrate-responsive lateral gate which is open toward the inner leaflet of the membrane but closes upon drug binding. Together, our results suggest a modified model for the functional conformational cycle of MdfA that does not invoke canonical elements of alternating access.

Keywords

MdfA; Multidrug transport; Conformational cycle; DEER; Nanodiscs

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Introduction

Multidrug (Mdr) transporters play important roles in protection of living cells against chemically and structurally diverse cytotoxic compounds. Bacterial Mdr transporters of the Major Facilitator Superfamily (MFS), which is probably the largest group of transporters [1], usually operate as drug/H⁺ antiporters [2]. This group includes MdfA from *E. coli*, a relatively well characterized Mdr transporter [3]. MdfA consists of 410 amino acid residues organized in 12 transmembrane helices (TM) with both N- and C- termini located in the cytoplasm [4]. An initial 3D model for MdfA [5] has received direct support from high-resolution crystal structures [6, 7] that reveal an overall architecture similar to MFS transporters [8], including the MFS fold with two domains related by twofold pseudosymmetry and consisting of six consecutive TMs.

In canonical transport models, the transporter cycles through four steps [8] that involve at least three different conformations, inward open (I_o), outward open (O_o), and occluded. In the inward and outward open states, one side of the transporter is closed to avoid leaks that would short ion gradients. The occluded state, where the inside and outside sides are simultaneously closed (I_c/O_c), can potentially have multiple conformations depending on the occupancy of its ligand binding site(s) and/or side chain protonation.

Unlike substrate-specific MFS transporters that display relatively defined conformational states during transport [8], Mdr-MFS transporters, including MdfA, are expected to be more flexible in order to populate transport-competent conformations that fit a variety of chemically and structurally dissimilar substrates. Recent crystal structures of MdfA captured two conformations termed outward open (O_o) and inward facing (I_f) [6, 7]. The O_o conformation is stabilized by a Fab fragment bound at the cytoplasmic face of wild-type MdfA. In the I_f conformation, the crystallized mutant MdfA(Q131R) contains a ligand in the proposed Mdr-recognition pocket and its cytoplasmic face is minimally open. Although revealing the molecular architecture of the transporter, these structures neither inform how alternating access is executed nor do they establish its drug/ion dependence. In addition, other conformations are likely populated as predicted from the classical alternating access of MFS transporters [8–11].

In order to address these questions and illuminate the conformational dynamics underpinning transport, we utilized double electron electron resonance (DEER) distance measurements [12] between spin labels attached to edges of TMs on the cytoplasmic and periplasmic sides of nanodisc-reconstituted MdfA. The obtained distances are discussed in comparison to those calculated from the two crystal structures [6, 7] and in light of similar DEER studies of MdfA in detergent solution [13]. DEER analysis was complemented with cysteine cross-linking studies designed to detect changes in proximity of helices. Cysteines were inserted at relevant positions in MdfA and tested for disulfide bridge formation in the native membrane environment under various conditions, as recently reported for other MdfA variants [14]. Together, our results reveal that in its resting state, apo MdfA assumes a flexible conformation that on average is relatively closed on both sides of the membrane with a putative lateral gate open to the inner leaflet of the membrane. In the presence of the

cationic substrate tetraphenylphosphonium (TPP), MdfA assumes an occluded conformation where both the cytoplasmic side and the lateral gate of MdfA are constricted, as well as the periplasmic gap between TM1 and TM7. Protonation did not lead to large-scale conformational changes. To our knowledge, this is the first report of a MFS transporter mechanism that does not involve large amplitude conformational changes as predicted by classical alternating access models.

Results and discussion

General methodology

MdfA couples the downhill movement of protons in exchange for cytotoxic molecules. Therefore, to identify conformations sampled in the transport cycle, we investigated structural rearrangements of MdfA utilizing: (i) DEER distance measurements of spin labeled mutants in *E. coli* lipid-containing nanodiscs under various conditions, including binding of the substrate TPP and protonation, and (ii) cysteine cross-linking of MdfA in native membranes. For both aims, double cysteine mutants of MdfA were constructed and tested for their expression and multidrug resistance activity *in vivo* (Figure S1). Only functional double cysteine mutants were selected for the studies below. Notably, the following pairs, TM1⁴¹-TM7²⁴³, TM5¹⁶²-TM8²⁵⁴ and TM2⁷³-TM11³⁴⁷, which reside on the periplasmic face of MdfA, were slightly less active in resistance assays compared to other mutants, possibly due to disulfide bonds that may have formed spontaneously under the oxidizing conditions of the periplasmic environment.

MdfA variants were purified and labeled in detergent with the nitroxide spin label ((1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate) MTSSL [13]. The spin labeled proteins were then further purified by size exclusion chromatography (SEC) (Figure S2). Spin-labelled MdfA variants were reconstituted into nanodiscs containing *E. coli* polar lipids and the distance distribution between the labels in each MdfA variant was obtained by DEER measurements. For some of the samples, the distributions contained components that generally arise from aggregation (indicated by * in the Figures below) although these often broad components could also reflect the flexibility of the backbone.

The presumed aggregation components were neither linked to the type of detergent used for extraction from native membranes (Figure S3) nor did they directly correlate with the heterogeneity of the SEC profile (Figure S2). Generally, these components were well separated from sharper short-distance components. Therefore, the following discussion of the distance distributions will focus on the latter, as these arise most likely from discrete conformations and therefore have the most potential to correspond to the crystal structures. Confidence band analysis of these distributions (Figure S3, Figure S5 and Figure S6) demonstrate that the sharper components are well defined in position and width.

The cytoplasmic side of nanodisc-reconstituted MdfA is closed

Previous observations have shown that the cytoplasmic side of MdfA in cell membranes is relatively closed [14]. To examine whether this finding holds for nanodisc-reconstituted MdfA, we chose functional cysteine pairs located at the cytoplasmic edges of TMs helices

for spin labeling (Figure 1(a) and 1(b)). For each of the pairs, the derived distance distribution and the expected distance distributions for the I_f and O_o crystal structures are compared in (Figure 1, Figure S4 and Table 1). Under conditions that favor deprotonation, we observed that the average distances for all pairs, TM1²⁰-TM8²⁸⁰ (24.2 Å), TM2⁷¹-TM8²⁷⁷ (45.3 Å), TM4¹³⁴-TM10³⁴¹ (27.9 Å), TM4¹³⁴-TM11³⁴⁸ (24.8 Å), TM5¹³⁷-TM8²⁷⁷ (19.9 Å), TM5¹³⁷-TM10³⁴¹ (24.3 Å), and TM5¹⁴⁰-TM11³⁴⁸ (23.8 Å) are similar to or shorter than those predicted from the I_f structure (26.4, 47.3, 33.5, 24.1, 20.0, 27.1, and 22.8 Å, respectively). When compared to the O_o crystal structure, three pairs exhibited DEER-derived distances that are similar or slightly shorter (TM2⁷¹-TM8²⁷⁷ (45.3 Å), TM4¹³⁴-TM11³⁴⁸ (28.3 Å), TM5¹⁴⁰-TM11³⁴⁸ (24.5 Å)). All other pairs resulted in longer distances (TM1²⁰-TM8²⁸⁰ (19.8 Å), TM4¹³⁴-TM10³⁴¹ (24.9 Å), TM5¹³⁷-TM8²⁷⁷ (17.3 Å), TM5¹³⁷-TM10³⁴¹ (17.7 Å)) (Figure S4), two of which involve TM5 (see discussion of TM5 behavior below). This suggests that the cytoplasmic side of reconstituted MdfA is relatively closed, although moderately less than in the O_o crystal structure, in agreement with our previous studies [14].

The periplasmic side of MdfA in nanodiscs and cell membrane is closed

Similarly, to examine the periplasmic side of MdfA in nanodiscs, we selected functional variants with cysteine pairs located at the edges of TMs for spin labeling (Figure 2(a) and (b)). For each of the pairs, we obtained distance distributions at pH 7.5 or 9.0, where the protein is deprotonated [15]. The DEER-derived distances (Figure 2, Figure S4 and Table 1) were fairly comparable to the I_f crystal structure, with minor deviations toward longer distances for mutants TM1⁴⁶-TM7²⁴⁶, TM2⁵³-TM8²⁵⁹, TM3¹⁰¹-TM11³⁷³, TM5¹⁶³-TM7²⁴⁵, and TM5¹⁶⁸-TM11³⁷³, while mutant TM2⁵³-TM5¹⁶³, TM2¹⁶³-TM5³⁰⁷, TM5¹⁶³-TM11³⁷³, and TM8²⁵⁹-TM11³⁷¹ deviated to shorter distances.

In contrast to our previous results with DDM-solubilized MdfA that suggest an open periplasmic side [13], the distance distributions of most reconstituted periplasmic variants (with the exception of TM2⁵³-TM8²⁵⁹) were shorter than those predicted from the O_o crystal structure (Figure 2, Figure S4 and Table 1). The DEER data suggest that lipids may stabilize a closed conformation on the periplasmic side. Importantly, this is in contrast with the expectation of an outward open conformation for deprotonated MdfA on the basis of a canonical antiport model.

The suggestion that MdfA assumes an outward closed (O_c) conformation is intriguing in light of our previous observations that the cytoplasmic side of MdfA in cell membranes [14] and lipid nanodiscs is also relatively closed (Figure 1 and Figure S5). A comparison between the O_o (Figure 3(a)) and I_f (Figure 3(b)) crystal structures shows that, as expected, periplasmic TM edges are closer to each other in the I_f conformation. Therefore, to test the proposed O_c conformation we designed the double cysteine mutants TM1⁴¹-TM7²⁴³, TM2⁵⁵-TM11³⁶⁹, TM5¹⁶²-TM8²⁵⁴ and TM5¹⁶²-TM8²⁵⁸ (Figure 3(a)) and (Figure 3(b)), and investigated their ability to form disulfide bonds as described recently [14]. TM5¹⁶³-TM7²⁴⁵, which according to both crystal structures should not form disulfide bonds (Figure 3(e)), served as a negative control. Disulfide bonds are manifested on SDS-PAGE by changes in the compactness of proteins which limit their denaturation under SDS conditions

leading to faster migration on the gel. The results show that TM1⁴¹-TM7²⁴³, TM2⁵⁵-TM11³⁶⁹, TM5¹⁶²-TM8²⁵⁴, and TM5¹⁶²-TM8²⁵⁸ formed cross-links spontaneously either *in vivo* or during cell disruption and fractionation (Figure 3(c) and Figure 3(d)). Cysteine residues in close proximity at the periplasmic side of MdfA may indeed form spontaneous disulfide bonds in the oxidizing periplasmic environment (Figure 3(c) and Figure 3(d)). Addition of CuCl₂, which catalyzes disulfide bond formation between very close cysteines, stimulated all the cross-linking reactions. In contrast, the control pair TM5¹⁶³-TM7²⁴⁵ did not form a disulfide bond even in the presence of CuCl₂ (Figure 3(f)).

Thus, EPR and cross-linking results suggest that deprotonated membrane-embedded MdfA assumes an O_c/I_c conformation (i.e. relatively closed on both sides of the membrane). In light of the previously observed predominantly outward facing conformation of MdfA in detergent micelles, we propose that the O_c/I_c conformation is stabilized by lipids. Such a conformation raises the question of how does MdfA recruit substrates? One possibility is that protonation or interaction with a substrate may induce conformational rearrangements of MdfA.

Protonation of MdfA in nanodiscs does not induce global conformational changes

To address this question, DEER distance measurements were performed at pH 5 to deprotonate acidic residues critical for transport. Figure 4, Figure S5, and Figure S6 shows that in most cases, the effect of low pH was marginal and several of the variants showed only minor changes in the distance distributions often manifested as a shift in the ratio of distance components (e.g. TM8²⁵⁹-TM11³⁷¹, TM2⁷¹-TM8²⁷⁷, TM4¹³⁴-TM11³⁴⁸, and TM5¹⁴⁰-TM11³⁴⁸). Only one pair, where MdfA was labeled at the periplasmic edges of TM1⁴⁶-TM7²⁴⁶, reported a relatively large pH-dependent distance change from 30 Å at pH 7.5 to about 20 Å at pH 5, resulting in an even shorter distance than that calculated from the I_f crystal structure (26.5 Å) (Figure 4(a), Figure S6 and Table 1). Notably, TM1 and TM7 are kinked at their periplasmic edges in the crystal structures (Figure 4(b)), implying that the observed closure of these helices could involve changes in the kinks or movements of the entire helices (see below). The results with TM1 and TM7 suggest that at acidic pH, the periplasmic side of MdfA is even more tightly closed (see next section), possibly as a result of the protonation of the essential D34 residue [15].

Substrate-induced conformational changes in nanodisc-reconstituted MdfA

To probe the substrate-induced conformational changes, we determined distance distributions in the presence of the transport substrate TPP, which in detergent solution, MdfA binds with a *K_d* of ~ 5 μM [15, 16]. The results show that on the periplasmic side, TPP-mediated changes in distances were observed only between edges of TM3¹⁰¹-TM11³⁷³ and TM1⁴⁶-TM7²⁴⁶ (Figure 5(a), Figure S6 and Table 1). A TPP-mediated opening on the periplasmic side has been observed in a recent smFRET study [17]. On the cytoplasmic side, TPP addition shortens the distances between TM5¹³⁷-TM10³⁴¹ and TM1²⁰-TM8²⁸⁰. A marginal shift in the distance populations of TM5¹⁴⁰-TM11³⁴⁸ is also noted (Figure 5(b), Figure S5 and Table 1).

The observations that the periplasmic edges of TM1⁴⁶ and TM7²⁴⁶ exhibit unique responses to low pH and TPP suggest involvement in ligand-induced conformational changes. However, the distance between the labels on these helices is near the shorter limit of DEER measurements which could obscure the scale of the movement. Therefore, we tested whether cysteines at sites TM1⁴⁶ and TM7²⁴⁶ (Figure 6(a), red spheres) can form a disulfide bond. Notably, according to the crystal structures, TM1 and TM7 are both kinked in opposite directions at the periplasmic side of the membrane, suggesting that they could rotate to close the gap between their edges and form a disulfide bond. Therefore, we also tested two, closer cysteines at TM1⁴¹ and TM7²⁴³, located at the kink sites of these helices as a positive control (Figure 6(a), blue spheres). The results, shown in (Figure 6(b)), demonstrate efficient cross-linking between TM1⁴¹ and TM7²⁴³, as might be expected. Surprisingly however, partial cross-linking was also detected between TM1⁴⁶ and TM7²⁴⁶ which was enhanced by TPP (Figure 6(c)). These observations support the notion that the edges of the kinked TM1 and TM7 respond to TPP binding possibly by twisting toward each other, thereby further closing part of the periplasmic side of MdfA.

Together, the DEER and cross-linking studies show that the substrate-induced conformational changes on both sides of the transporter are rather limited except for the apparent closure between the edges of TM1 and TM7. Therefore, we propose that upon TPP binding, MdfA acquires a substrate-occluded conformation, which is sealed on the periplasm by the edges of TM1 and TM7. Intriguingly however, the results obtained with apo-MdfA or under conditions of low pH or in the presence of TPP do not illuminate how MdfA recruits substrates from the cytoplasm in an O_c/I_c conformation.

Evidence for a lateral substrate-responsive gate in MdfA in native membranes

One possibility would be that the substrate recruitment and release involve transient conformations. For lipophilic substrates, another possibility is that they may bind MdfA through an opening in the transporter that is exposed to the inner leaflet of the membrane. This concept was invoked for Mdr transporters, the so called hydrophobic cleaner model [18, 19]. Thus, a lateral opening(s) towards this leaflet of the lipid bilayer is highly likely for binding of hydrophobic substrates.

We hypothesize that such an opening(s) is theoretically plausible between TMs 5 and 8 (Figure 7(a)) and/or 2 and 11 (Figure 7(e)), the only peripheral TM pairs that directly separate the recognition pocket of MdfA from the membrane. Since the distances between neighboring residues in these TM pairs in the crystal structures are in the range of 1–1.5 nm, we tested this hypothesis using cysteine cross-linking. We focused on regions in the cytoplasmic halves of TM5/TM8 and TM2/TM11, which may constitute a gate that opens toward the inner leaflet of the membrane: TM5¹⁴⁰-TM8²⁷⁶, TM5¹⁴⁴-TM8²⁷² (Figure 7(a), red spheres) and TM2⁷³-TM11³⁴⁷ (Figure 7(e), yellow spheres). Unlike the cross-links that may fully form spontaneously in the periplasmic side (Figure 3), we did not expect spontaneous cross-links between cysteine pairs located at the cytoplasmic side of MdfA. Membranes prepared from cells expressing the various double cysteine mutants were incubated with or without TPP, followed by incubation with or without CuCl₂ and then analyzed by Western blotting. The results show that the cytoplasmic edges of TM2⁷³ and

TM11³⁴⁷ are fully cross-linked in the absence or presence of the substrate TPP (Figure 7(f)). In contrast, the relatively inefficient cross-linking between TM5¹⁴⁰ and TM8²⁷⁶ or between TM5¹⁴⁴ and TM8²⁷² is enhanced by addition of TPP (Figure 7(b) and Figure 7(c)).

Analysis of the kinetics of cross linking between TM5¹⁴⁴ and TM8²⁷² indicate that the reaction is slow and TPP promotes full cross-linking after 30 min (Figure 7(d)). These results confirm that a lateral gate may exist for TPP recruitment between the cytoplasmic halves of TM 5 and TM8. Furthermore, upon TPP binding, a putative inner-leaflet oriented lateral gate between TM5 and TM8 closes, and as proposed above, the substrate becomes occluded prior to its release on the outside.

Conclusion

The investigation reported here was designed to test the notion that there is a general mechanism of alternating access in Mdr-MFS transporters. Indeed, the selection of spin label pairs was guided by previous DEER analysis of another Mdr-MFS transporter, LmrP [20]. Our results, which offer a view of the conformational behavior of MdfA in a lipid environment, deviate in important aspects from the accepted paradigm of alternating access. In contrast to the expected large amplitude distance changes upon protonation, as observed in the cases of LmrP [21] and LacY [22, 23], our results suggest a distinct mechanism that could have general implications for a subfamily of Mdr-MFS transporters. Although changing the pH effects protonation/deprotonation of acidic residues, it does not mimic the physiological situation of a pH gradient where the cytoplasmic and periplasmic regions are exposed to different pHs. Moreover, the membrane potential may further shift the energetics of the equilibria between conformations or conceivably stabilize yet unidentified conformations.

As depicted in Fig. 8, which summarizes our conclusions from this study, the resting state of MdfA in the membrane is O_c/I_c (state 1). Presumably in this state, MdfA recruits a lipophilic drug (LD) from the inner leaflet of the membrane (state 2) through a membrane accessible portal between TMs 5 and 8. Even in its I_c conformation, MdfA is sufficiently open to allow diffusion of less hydrophobic drugs (hydrophilic drug; HD) from the cytoplasm into its Mdr-recognition pocket, possibly facilitated by non-rigid body movements of TM4 and TM10 relative to each other [14]. Because we found no evidence of large-scale conformational changes that could underpin such a mechanism, we propose that drug recruitment from the cytoplasm involve sparsely populated inward-open conformations possibly implied by the broad distance distributions. The results suggest that upon substrate recruitment, MdfA persists in an occluded conformation (state 3), wherein the periplasmic edges of helices TM1 and TM7 move toward each other. In parallel, the cytoplasmic edges of TM5 and TM8 as shown here as well as TM4 and TM10 (shown previously in [14]), also move toward each other. MdfA is expected then to open on the periplasmic side to release the substrate while simultaneously binding a proton at site D34 (state 4). This state, we surmise, is transient and may have been captured in the O_o crystal structure [7]. Because the protons are translocated to the cytoplasm, a proton pathway must exist through the transporter although it is not associated with large structural changes.

Our model deviates from a previous one [15] in mechanistically important aspects. The current model proposes that the resting state of MdfA is O_c/I_c and importantly, deprotonated. Thus, substrates that bind MdfA from the cytoplasm or the inner leaflet of the membrane do not have to compete with protons. Physiologically, this scenario is plausible because the slightly open cytoplasmic side of MdfA in that conformation is exposed to a cytoplasmic pH of ~7.6 [24]. It is thus conceivable that protonation is only needed for reorienting MdfA, after which the proton is released into the cytoplasm. Another important difference is that according to our cross-linking results, MdfA may have a lateral gate (state 1 and 2) exposed to the inner leaflet of the membrane in its resting state. This gate closes upon substrate binding. Finally, our studies also revealed small scale inter-TMs movements that are uncoupled from the conceptual rigid-body movement of the two halves of MdfA against each other, according to the alternating access model. The inter-TMs movements may play a role in accommodating chemically dissimilar substrates.

Methods

Strains, plasmids, and mutants

All experiments were conducted with *E. coli* UTL2 [25] derivative (UTL2 $mdfA::Kan$). Plasmid pT7-5/200us/*CL-mdfA-6His* was used for resistance, expression and crosslinking experiments. Plasmid pUC18/Para/*CL-mdfA-6His* was used for overexpression and purification [16, 26–29]. All mutants were generated utilizing standard PCR methods with mutagenic oligonucleotide primers and plasmids encoding cysteine-less (CL) MdfA-6His as a template. All plasmids were sequenced to verify that only the desired mutations were inserted.

Protein Expression Analysis

Overnight cultures of *E. coli* UTL2 $mdfA::Kan$ harboring empty vector or expressing CL-MdfA or variants were diluted 1:100 and grown to A_{600} of 0.8–1.2. Cells were harvested and resuspended in buffer A (20 mM Tris-HCl, 500 mM NaCl, 10% glycerol), supplemented with 1 mM PMSF. Cells were sonicated and cell debris was removed by centrifugation. The supernatant was then centrifuged at 244,000 g for 30 min to pellet the membranes. Membranes were resuspended in Buffer A and the total membrane protein concentration was analyzed by Lowry. Samples (20 μ g of total membrane protein) were then subjected to 12.5% (w/v) SDS/PAGE, electroblotting, and detection using India HisProbe-horseradish peroxidase (Thermo Scientific) and ECL.

Drug resistance assay

Multidrug resistance was assayed as described [29]. Briefly, *E. coli* UTL2 $mdfA::Kan$ harboring empty vector or expressing MdfA variants were grown aerobically to A_{600} of ~1 and a series of 10-fold dilutions was prepared. 4 μ l of the serial dilutions were spotted on drug-containing LB-agar plates and tested for growth after overnight incubation at 37°C.

MdfA overexpression and purification for biochemical studies

E. coli cells harboring plasmid pUC18/Para/*CL-mdfA-6His* or any of the mutated constructs were grown at 37°C in terrific broth [30] medium supplemented with ampicillin (200

µg/mL) and Kanamycin (30 µg/mL). Cultures were diluted 1:1000, grown to ~4 A_{600} units, and induced with 0.2% (w/v) arabinose for 1.5 h at 37°C. Cell pellets were resuspended in 50 mM KPi pH 7.3 supplemented with 2 mM MgSO₄, 10 µg/mL DNase (Sigma) and 1 mM PMSF and passed three times through a pressure cell homogenizer (Stansted) at 15 kPsi for disruption. Cell debris was removed by centrifugation (15 min, 20,000 g), and the membranes were collected by ultracentrifugation (1 h, 167,000 g). The membranes were suspended by homogenization in solubilization buffer (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 10% glycerol, 5 mM Imidazole), supplemented with 2 mM β-mercaptoethanol, snap-frozen in liquid nitrogen and stored at -80 °C.

For MdfA purification, thawed membranes were solubilized by addition of 20% n-dodecyl-β-D-maltopyranoside (DDM, Anatrace) to a final concentration of 1.1% and incubation at 4°C for 2 h. When indicated, n-dodecyl-α-D-maltopyranoside (αDDM, Anatrace) was used instead of βDDM. Insoluble material was discarded by ultracentrifugation (30 min, 167,000 g) and the soluble fraction was mixed with solubilization buffer-equilibrated *Talon* beads (Clontech) (typically 0.5 mL bed volume for 2.5 g of thawed membranes). Next, the mixture was agitated for 2 hr at 4°C and the suspension was poured into a column and washed with 30 column volumes (CVs) of solubilization buffer. MdfA was eluted in 20 mM Tris-HCl pH 7.2, 0.12 M NaCl, 10% glycerol, 0.1% DDM, 200 mM imidazole.

Lipid Preparation

E. coli polar lipids (Avanti Polar Lipids) were dissolved in chloroform:methanol (3:2), dried under vacuum, and then placed in a desiccator overnight, under vacuum. Lipids were then hydrated by addition of 20 mM Tris-HCl, pH 7.5, 0.12 M NaCl (15 mg/ml) and subjected to 10 cycles of freezing in liquid nitrogen and thawing, followed by sonication in a bath sonicator. Lipids were flash frozen and stored as aliquots in -80°C.

Membrane Scaffold Protein 1D1E3 (MSP1D1E3) purification

MSP1D1E3 was prepared as described previously [31]. Briefly, *E. coli* BL21 (DE3) harboring plasmid pET-28a(+) with MSP1D1E3 were grown overnight at 37°C in TB medium supplemented with Kanamycin (30 µg/mL). Cultures were diluted 1:100 into 1 L of TB, supplemented with Kanamycin (30 µg/mL), grown at 37°C to 2.5–3.0 A_{600} units, induced by addition of 1 mM IPTG and grown for additional 4 h at 37°C. Cells were harvested, resuspended in lysis buffer (20 mM sodium phosphate, pH 7.4; 1% Triton X-100), supplemented with 1 tablet of Complete Mini, EDTA-free Protease Inhibitor Cocktail (Roche) and 1 mM PMSF. Cells were sonicated twice at 30% amplitude for 8 min (15 sec on, 15 sec off), the lysate was centrifuged at 30,000 g for 30 min, and the supernatant was filtered by a 0.2 µm filter. The supernatant was then mixed with preequilibrated Ni-NTA resin, incubated with rocking for 1 h, at 4°C, loaded onto a gravity column and the flow-through was discarded. The resin was then washed with 5 CVs of MSP wash 1 buffer (40 mM Tris-HCl, pH 8.0; 300 mM NaCl; 1% Triton X-100), 5 CVs of MSP wash 2 buffer (40 mM Tris-HCl, pH 8.0; 300 mM NaCl; 50 mM Sodium cholate), and 5 CVs of Ni-NTA buffer A (40 mM Tris-HCl, pH 8.0, 300 mM NaCl, 30 mM imidazole). The protein was then eluted step-wise with Ni-NTA buffer B (40 mM Tris-HCl, pH 8.0; 300 mM NaCl, 300 mM Imidazole). The eluted protein was passed through a HisTrap desalting

column (GE Healthcare) with 20 mM Tris-HCl, pH 7.5, 0.12 M NaCl. The protein was then concentrated using an Amicon Ultra-15 centrifugal unit (Merck), with a cutoff of 10 kDa, to A_{280} of 20–25.

Labeling with MTSSL and reconstitution into nanodiscs

Labeling was done by adding 20-fold molar excess of MTSSL (stock of 200 mM in DMF), followed by incubation at room temperature (RT) for 1.5 h, after which another 20-molar excess of MTSSL was added, with an additional incubation at RT for 1 h. In cases where large aggregation was seen, incubation was done at 4°C. The protein was then kept on ice at 4°C overnight (ON). The labeled protein was then concentrated using an Amicon Ultra-4 Centrifugation Unit (Merck), with a cutoff of 100 kDa, and loaded onto a Superdex 200 10/30 gel filtration column (GE Healthcare). The protein was eluted with gel filtration buffer (20 mM Tris-HCl, pH 7.5, 0.12 M NaCl, 10% glycerol, 0.03% DDM) and selected fractions were concentrated with an Amicon Ultra 0.5 Centrifugal Filters (Merck), with a cutoff of 100 kDa. The concentrated protein was then mixed with MSP1D1E3, lipids and sodium cholate at the following ratios: MdfA: MSP1D1E3, 1:10; MSP1D1E3: lipids, 1:60; lipids: detergent (DDM+sodium cholate), 1:5. The mixture was rocked gently for 1 h at 4°C, followed by two additions of BioBeads SM-2 (Biorad) (100 mg/1 ml mixture) and 1 h incubations. BioBeads (200 mg/1 ml) were added again and the mixture was incubated ON at 4°C with rocking, followed by another addition of BioBeads (200 mg/1 ml) and 1 h incubation. BioBeads were removed by low-speed centrifugation, the mixture was filtered and loaded onto Superdex 200 10/30, and the nanodisc-reconstituted MdfA was eluted with the nanodisc buffer (20 mM Tris-HCl, pH 7.5, 0.12 M NaCl, 10% glycerol). Reconstitution was verified by SDS-PAGE and desired fractions were combined and concentrated using Amicon Ultra 0.5 Centrifugal Filters (Merck), with a cutoff of 100 kDa, to a spin-label concentration of 50–90 μ M (determined by Continuous wave (CW-EPR)).

Preparation of samples for DEER measurements

The concentration of glycerol in the samples was adjusted to 23.78% (v/v) before the preparation of different conditions (other than apo, pH 7.5). For the Apo, pH 5.0 condition, pH was adjusted by addition of 200 mM MES hydrate to a final concentration $\sim$ 40 mM. For the Apo, pH 9.0 condition, pH was adjusted by addition of 500 mM Bis-Tris propane to a final concentration of $\sim$ 47 mM. In both cases, the pH of the samples was verified by using a pH-indicator paper. For the TPP, pH 7.5 condition, 10 mM of TPP was added to a final concentration of 1–2 mM. Samples were then loaded onto 1.1×1.6 mm capillaries and flash-frozen in liquid nitrogen.

DEER measurements

DEER measurements were carried out at Q-band (34 GHz) on two E580 instruments (Bruker) using standard dead-time free, four pulse sequences [32]. The observer $\pi/2$ pulse lengths were 10 or 12 ns at 10W and 12 or 16 ns at 40W. The pump pulse lengths were 40 ns at both powers with a 62 MHz separation between pulses. Traces were collected at 83 K.

The software used in the analysis of the DEER traces is an upgrade to a previously used version [31] that implements a number of well-established approaches to DEER data

analysis [33–35]. In this software the distance distribution is assumed to consist of a sum of Gaussians. The depth of modulation and slope were allowed to vary for each condition. The reduced chi-square between the data and fit was minimized using the trust-region-reflective algorithm implemented in the MATLAB routine 'lsqnonlin'. The statistical significance between fits with increasing number of Gaussian distributions was determined using the BIC criteria, while the confidence bands for the distance distributions were determined using the delta method [36].

Cysteine cross-linking

Overnight cultures of UTL2*mdfA::kan* expressing double cysteine MdfA mutants [in a cysteine-less (CL) background] [37] were grown at 37°C to 1.0–1.5 A₆₀₀ units. Cells were harvested, resuspended in 50 mM sodium-HEPES buffer (pH 8), supplemented with 1 mM dithiothreitol (DTT). Membranes were prepared and collected as described above, and resuspended in sodium-HEPES buffer (without DTT) at a protein concentration of ~10 µg/mL. Oxidative cross-linking with CuCl₂ was carried out with aliquots of membrane suspension (40 µg in 40 µL of the sodium-HEPES buffer). Membranes were preincubated for 20 min with or without 5 mM TPP, followed by addition of 0.5 mM CuCl₂ (stock of 5 mM). Membranes were incubated with CuCl₂ and the reaction was terminated by addition of 10 mM EDTA in a non-reducing protein sample buffer, pH 8. Samples (~12.5 µg of total membrane proteins) were then subjected to various versions of SDS/PAGE and ran at low voltage at 4°C until the 35 kDa marker. Gels were then analyzed with Western blot, using India HisProbe-horseradish peroxidase (Thermo Scientific).

For mutants CL-MdfA(L41C/P243C), CL-MdfA(P55C/K369C), CL-MdfA(A162C/S254C), and CL-MdfA(A162C/G258C) (Figure 3(c) and Figure 3(d)), crosslinking was performed at 30°C for 40 min. Samples were subjected to 15% SDS-PAGE and ran at 50 v at 4°C until the 35 kDa marker.

For CL-MdfA(A163C/I245C) (Figure 3(f)), crosslinking was performed at 30°C for 40 min. Samples were subjected to 10% SDS-PAGE and ran at 30 v at 4°C until the 35 kDa marker.

For CL-MdfA(L41C/P243C) (Figure 6(b)), membranes were incubated with TPP at 4°C for 20 min, followed by addition of CuCl₂ and incubation at 4°C for 0–8 min. Samples were subjected to a 4%–8% (1ml)–10% (6.4ml) Tris Tricine SDS-PAGE and ran at 50 v at 4°C until the 35 kDa marker.

For CL-MdfA(Q46C/I246C) (Figure 6(c)), membranes were incubated with TPP at 4°C for 20 min, followed by addition of CuCl₂ and incubation at 4°C for 0–8 min. Samples were subjected to 15% SDS-PAGE and ran at 80 v at 4°C until the 35 kDa marker.

For CL-MdfA(I140C/A276C) (Figure 7(b)), membranes were incubated with TPP at RT for 20 min, followed by addition of CuCl₂ and incubation at 25°C for 10 min. Samples were subjected to 15% SDS-PAGE and ran at low voltage at 4°C until the 35 kDa marker.

For CL-MdfA(A144C/N272C) (Figure 7(c) and Figure 7(d)), membranes were incubated with TPP at RT for 20 min, followed by addition of CuCl₂ and incubation at 25°C for 5–40

min. Samples were subjected to 12.5% SDS-PAGE and ran at low voltage at 4°C until the 35 kDa marker.

For CL-MdfA(G73C/G347C) (Figure 7(f)), membranes were incubated with TPP at RT for 20 min, followed by addition of CuCl₂ and incubation at 25°C for 10 min. Samples were subjected to 12.5% SDS-PAGE and ran at low voltage at 4°C until the 35 kDa marker.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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- We investigated the conformational states of the multidrug transporter MdfA, embedded in a lipid bilayer.
- We found that the resting state of MdfA is a flexible outward-closed/inward-closed conformation.
- We identified substrate-induced movements between TMs that are uncoupled from the conceptual rigid-body movements in MFS transporters.
- We describe a putative lateral gate that allows the recruitment of lipophilic drugs from the inner leaflet of the membrane.
- We conclude that MdfA does not follow the canonical rocker-switch models of MFS transporters.

Figure 1. Distance distributions for pairs on the cytoplasmic side of MdfA.

A cytoplasmic view of MdfA in the (a) I_f (pdb 4ZP0) and (b) O_o (pdb 6GV1) crystal structures. Residues that were labeled with MTSSL are marked as red spheres and pairs of residues are connected by red lines. (c - i). Distance distributions of the apo protein were measured at pH 7.5 (blue) and 9.0 (orange). The MMM-derived distance distributions, calculated using the crystal structures 4ZP0 (I_f , shaded purple) and 6GV1 (O_o , shaded green), overlaid with the DEER derived distance distributions at pH 7.5 (blue) and pH 9.0 (orange) [12]. Mutant pairs are indicated above their corresponding graphs. Mutant $TM1^{20}$ - $TM8^{280}$ (i), was solubilized with α -DDM. Asterisks indicate peaks resulting from partial aggregation observed in some samples.

Figure 2. Distance distributions for pairs on the periplasmic side of MdfA.

A periplasmic view of MdfA in the (a) I_f (pdb 4ZP0) and (b) O_o (pdb 6GV1) crystal structures. Residues that were labeled with MTSSL are marked as red spheres and pairs of residues are connected by red lines. (c - k). Distance distributions of the apo protein were measured at pH 7.5 (blue) and 9.0 (orange). The MMM-derived distance distributions, calculated using the crystal structures 4ZP0 (I_f , shaded purple) and 6GV1 (O_o , shaded green), overlaid with the DEER derived distance distributions at pH 7.5 (blue) and pH 9.0 (orange) [12]. Mutant pairs are indicated above their corresponding graphs. Asterisks indicate peaks resulting from partial aggregation observed in some samples.

Figure 3. Disulfide bond formation on the periplasmic side of a cell membrane-embedded MdfA. (a - b). The periplasmic face of MdfA in the (a) O_o (pdb 6GV1) and (b) I_f (pdb 4ZP0) crystal structures. Cysteine insertions are marked in pink (TM2⁵⁵-TM11³⁶⁹), orange (TM1⁴¹-TM7²⁴³) and yellow (TM5¹⁶²-TM8²⁵⁴/TM8²⁵⁸) and the pairs are connected by red lines. (c - d). Membranes from cells expressing CL-MdfA (CL), (c) TM2⁵⁵-TM11³⁶⁹ and TM1⁴¹-TM7²⁴³, (d) TM5¹⁶²-TM8²⁵⁴ and TM5¹⁶²-TM8²⁵⁸, were incubated with or without CuCl₂, separated by a 15% SDS-PAGE and analyzed by Western blot. (e) The periplasmic face of MdfA in the I_f (pdb 4ZP0) conformation. Cysteine insertions are marked as red spheres. (f) Membranes from cells expressing CL-MdfA, or TM5¹⁶³-TM7²⁴⁵ were incubated with or without CuCl₂, separated by a 10% SDS-PAGE and analyzed by Western blot.

Figure 4. Protonation-induced distance changes of MdfA.

Distance distributions of the apo protein at pH 7.5 (blue) and 5.0 (brown) of mutants in the (a) periplasmic side and (c) cytoplasmic side of MdfA [12]. Mutant TM5¹⁴⁰-TM11³⁴⁸ was solubilized with α -DDM. Asterisks indicate peaks resulting from partial aggregation observed in some samples. (b) The periplasmic face of the I_F crystal structure of MdfA (pdb 4ZP0) is shown with TM1 and TM7 (orange segments) and residues 46 and 246 (blue spheres).

Figure 5. TPP-induced distance changes of MdfA.

Distance distributions measured at pH 7.5 of the apo protein (blue) and in the presence of TPP (purple) of (a) periplasmically- and (b) cytoplasmically-labeled MdfA [12]. Mutant TM5¹⁴⁰-TM11³⁴⁸ was solubilized with α -DDM. Asterisks indicate peaks resulting from partial aggregation observed in some samples.

Figure 6. Disulfide bond formation between TM1 and TM7 in a membrane-embedded MdfA. (a) The periplasmic face of MdfA in the *I_f* (pdb 4ZP0) crystal structures. TM1 and TM7 are shown in yellow and orange. The double-cysteine pairs TM1⁴¹-TM7²⁴³ and TM1⁴⁶-TM7²⁴⁶ are shown as blue and red spheres, respectively. (b - c) Membranes from cells expressing (b) TM1⁴¹-TM7²⁴³ and (c) TM1⁴⁶-TM7²⁴⁶, were incubated with or without TPP, followed by an additional incubation at 4°C, for 0–8 min, in the presence of CuCl₂. Samples were then separated on an SDS-PAGE and analyzed by Western blot.

Figure 7. Disulfide bond formation between cysteines inserted on both sides of a putative inner leaflet lateral gate(s) of MdfA.

The I_f (4ZP0, left panels) and the O_o (6GV1, right panels) crystal structures of MdfA with (a) TM5 and TM8 shown in blue and cysteine insertions marked as red spheres and (e) TM2 and TM11 shown in green and cysteine insertions marked as yellow spheres. Membranes from cells expressing (b) CL- MdfA (CL), TM5¹⁴⁰-TM8²⁷⁶, (c) CL-MdfA (CL), TM5¹⁴⁴-TM8²⁷², (d) TM5¹⁴⁴-TM8²⁷², or (f) TM2⁷³-TM11³⁴⁷ were incubated with or without CuCl₂, separated by (b) 15%, (c - d, f) 12.5% SDS-PAGE and analyzed by Western blotting.

Figure 8. Model for MdfA-catalyzed substrate/H⁺ antiport.

1. In the proposed resting state of membrane-embedded MdfA, the protein is deprotonated. Both the periplasmic and cytoplasmic sides are relatively closed. **2.** A lipophilic drug (LD) may enter the multidrug recognition pocket from the inner leaflet of the membrane, between TM5 and TM8, while less hydrophobic drugs (hydrophilic drugs, HD) may enter from the cytoplasm, between TM4 and TM10. **3.** Substrate binding triggers a subtle conformational change that further closes the cytoplasmic side and the periplasmic edges of TM1 and TM7, while slightly exposing the multidrug-binding pocket to the periplasm. **4.** Protons bind to MdfA once the substrate is released to the extracellular side. **5.** The proton-binding event triggers a conformational switch and proton-release recycles MdfA to the resting state.

Table 1.
Summary of all DEER-derived distances.

r_{avg} are the average distances, calculated without the broad component. S.D represent the standard deviation of the average value. All measurements are in Å.

	pH 5.0		pH 7.5		pH 9.0		TPP		I _f (pdb 4PZ0)		O _o (pdb 6GV1)	
	r_{avg} (Å)	S.D.	r_{avg} (Å)	S.D.	r_{avg} (Å)	S.D.	r_{avg} (Å)	S.D.	r_{avg} (Å)	S.D.	r_{avg} (Å)	S.D.
TM1 ^{2U} -TM8 ^{28U}	24.6	5.2	24.2	5.3	23.6	5.4	21.3	4.3	26.4	5.3	19.8	4.4
TM2 ⁷¹ -TM8 ²⁷⁷	44.1	9.4	45.3	10.0	44.3	9.9	44.8	10.2	47.3	3.0	45.3	3.2
TM4 ¹³⁴ -TM10 ³⁴¹	28.8	4.9	27.9	4.4	27.6	4.3	26.8	3.9	33.5	1.3	24.9	2.8
TM4 ¹³⁴ -TM10 ³⁴⁸	24.8	3.6	24.8	3.5	24.8	3.8	25.2	3.8	24.1	1.6	28.3	1.7
TM5 ¹³⁷ -TM8 ²⁷⁷	19.7	3.7	19.4	3.5	19.9	3.7	19.0	3.3	20.0	5.1	17.3	3.6
TM5 ¹³⁷ -TM10 ³⁴¹	25.3	4.3	24.3	4.0	24.4	3.7	23.0	3.9	27.1	3.8	17.7	4.2
TM5 ^{14U} -TM8 ³⁴⁸	24.4	3.1	23.8	3.4	23.3	3.2	23.1	3.6	22.8	4.8	24.5	0.7
TM1 ⁴⁶ -TM7 ²⁴⁶	25.2	5.9	28.4	5.6	29.3	5.2	26.7	5.3	26.5	3.3	38.2	3.2
TM2 ⁵³ -TM5 ¹⁶³	29.6	6.9	30.7	5.7	31.2	5.0	30.0	6.5	33.3	2.1	33.6	2.2
TM2 ⁵³ -TM8 ²⁵⁹	28.6	6.5	31.4	6.0	32.2	5.7	30.1	6.4	36.2	1.7	37.3	3.3
TM3 ^{1U1} -TM11 ³⁷³	32.1	4.5	34.1	5.4	37.0	5.4	40.0	3.2	31.9	2.3	45.3	3.3
TM5 ¹⁶³ -TM7 ²⁴⁵	23.3	5.1	23.1	5.0	23.4	5.3	22.8	4.9	19.1	2.8	34.3	3.5
TM5 ¹⁶³ -TM9 ^{3U7}	36.7	4.6	37.3	4.5	37.2	4.7	36.7	4.4	38.8	3.4	45.3	4.0
TM5 ¹⁶³ -TM11 ³⁷³	29.1	3.9	27.7	4.1	28.2	4.1	26.9	4.0	32.0	3.6	35.5	4.4
TM5 ¹⁶⁸ -TM11 ³⁷³	35.2	3.6	36.4	3.8	36.0	3.8	36.8	3.7	33.4	2.8	44.0	3.5
TM8 ²⁵⁹ -TM11 ³⁷¹	35.1	2.0	34.4	2.2	34.2	2.2	34.5	2.2	36.2	2.2	37.1	1.7