Mapping Daunorubicin-binding Sites in the ATP-binding Cassette Transporter MsbA Using Site-specific Quenching by Spin Labels

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ATP-binding cassette (ABC) transporters transduce the free energy of ATP hydrolysis to power the mechanical work of substrate translocation across cell membranes. MsbA is an ABC transporter implicated in trafficking lipid A across the inner membrane of Escherichia coli. It has sequence similarity and overlapping substrate specificity with multidrug ABC transporters that export cytotoxic molecules in humans and prokaryotes. Despite rapid advances in structure determination of ABC efflux transporters, little is known regarding the location of substrate-binding sites in the transmembrane segment and the translocation pathway across the membrane. In this study, we have mapped residues proximal to the daunorubicin (DNR)-binding site in MsbA using site-specific, ATP-dependent quenching of DNR intrinsic fluorescence by spin labels. In the nucleotide-free MsbA intermediate, DNR-binding residues cluster at the cytoplasmic end of helices 3 and 6 at a site accessible from the membrane/water interface and extending into an aqueous chamber formed at the interface between the two transmembrane domains. Binding of a nonhydrolyzable ATP analog inverts the transporter to an outward-facing conformation and relieves DNR quenching by spin labels suggesting DNR exclusion from proximity to the spin labels. The simplest model consistent with our data has DNR entering near an elbow helix parallel to the water/membrane interface, partitioning into the open chamber, and then translocating toward the periplasm upon ATP binding.

ATP-binding cassette (ABC) transporters transduce the energy of ATP hydrolysis to power the movement of a wide range of substrates across the cell membranes (1, 2). They constitute the largest family of prokaryotic transporters, import essential cell nutrients, flip lipids, and export toxic molecules (3). Forty eight human ABC transporters have been identified, including ABCB1, or P-glycoprotein, which is implicated in cross-resistance to drugs and cytotoxic molecules (4, 5). Inherited mutations in these proteins are linked to diseases such as cystic fibrosis, persistent hypoglycemia of infancy, and immune deficiency (6).

The functional unit of an ABC transporter consists of four modules. Two highly conserved ABCs or nucleotide-binding domains (NBDs) bind and hydrolyze ATP to supply the active energy for transport (7). ABCs drive the mechanical work of proteins with diverse functions ranging from membrane transport to DNA repair (3, 5). Substrate specificity is determined by two transmembrane domains (TMDs) that also provide the translocation pathway across the bilayer (7). Bacterial ABC exporters are expressed as monomers, each consisting of one NBD and one TMD, that dimerize to form the active transporter (3). The number of transmembrane helices and their organization differ significantly between ABC importers and exporters reflecting the divergent structural and chemical nature of their substrates (1, 8, 9). Furthermore, ABC exporters bind substrates directly from the cytoplasm or bilayer inner leaflet and release them to the periplasm or bilayer outer leaflet (10, 11). In contrast, bacterial importers have their substrates delivered to the TMD by a dedicated high affinity substrate-binding protein (12).

In Gram-negative bacteria, lipid A trafficking from its synthesis site on the inner membrane to its final destination in the outer membrane requires the ABC transporter MsbA (13). Although MsbA has not been directly shown to transport lipid A, suppression of MsbA activity leads to cytoplasmic accumulation of lipid A and inhibits bacterial growth strongly suggesting a role in translocation (14–16). In addition to this role in lipid A transport, MsbA shares sequence similarity with multidrug ABC transporters such as human ABCB1, LmrA of Lactococcus lactis, and Sav1866 of Staphylococcus aureus (16–19). ABCB1, a prototype of the ABC family, is a plasma membrane protein whose overexpression provides resistance to chemotherapeutic agents in cancer cells (1). LmrA and MsbA have overlapping substrate specificity with ABCB1 suggesting that both proteins can function as drug exporters (18, 20). Indeed, cells expressing MsbA confer resistance to erythromycin and ethidium bromide (21). MsbA can be photolabeled with the ABCB1/LmrA substrate azidopine and can transport Hoechst 33342 (H33342) across membrane vesicles in an energy-dependent manner (21).

The structural mechanics of ABC exporters was revealed from comparison of the MsbA crystal structures in the apo- and nucleotide-bound states as well as from analysis by spin labeling EPR spectroscopy in liposomes (17, 19, 22, 23). The energy har-
nessed from ATP binding and hydrolysis drives a cycle of NBD association and dissociation that is transmitted to induce reorientation of the TMD from an inward- to outward-facing conformation (17, 19, 22). Large amplitude motion closes the cytoplasmic end of a chamber found at the interface between the two TMDs and opens it to the periplasm (23). These rearrangements lead to significant changes in chamber hydration, which may drive substrate translocation (22).

Substrate binding must precede energy input, otherwise the cycle is futile, wasting the energy of ATP hydrolysis without substrate extrusion (7). Consistent with this model, ATP binding reduces ABCB1 substrate affinity, potentially through binding site occlusion (24–26). Furthermore, the TMD substrate-binding event signals the NBD to stimulate ATP hydrolysis increasing transport efficiency (1, 27, 28). However, there is a paucity of information regarding the location of substrate binding, the transport pathway, and the structural basis of substrate recognition by ABC exporters. In vitro studies of MsbA substrate specificity identify a broad range of substrates that stimulate ATPase activity (29). In addition to the putative physiological substrates lipid A and lipopolysaccharide (LPS), the ABCB1 substrates Ilmofosine, H33342, and verapamil differentially enhance ATP hydrolysis of MsbA (29, 30). Intrinsic MsbA tryptophan (Trp) fluorescence quenching by these putative substrate molecules provides further support of interaction (29).

Extensive biochemical analysis of ABCB1 and LmrA provides a general model of substrate binding to ABC efflux exporters. This so-called “hydrophobic cleaner model” describes substrates binding from the inner leaflet of the bilayer and then translocating through the TMD (10, 31, 32). These studies also identified a large number of residues involved in substrate binding and selectivity (33). When these crucial residues are mapped onto the crystal structures of MsbA, a subset of homologous residues clusters to helices 3 and 6 linking the putative substrate pathway (34). Consistent with a role in substrate binding and specificity, simultaneous replacement of two serines (Ser-289 and Ser-290) in helix 6 of MsbA reduces binding and transport of ethidium and taxol, although H33342 and erythromycin interactions remain unaffected (34).

The tendency of lipophilic substrates to partition into membranes confounds direct analysis of substrate interactions with ABC exporters (35, 36). Such partitioning may promote dynamic collisions with exposed Trp residues and nonspecific cross-linking in photo-affinity labeling experiments. In this study, we utilize a site-specific quenching approach to identify residues in the vicinity of the daunorubicin (DNR)-binding site (37). Although the data on DNR stimulation of ATP hydrolysis is inconclusive (20, 29, 30), the quenching of MsbA Trp fluorescence suggests a specific interaction. Spin labels were introduced along transmembrane helices 3, 4, and 6 of MsbA to assess their ATP-dependent quenching of DNR fluorescence. Residues that quench DNR cluster along the cytoplasmic end of helices 3 and 6 consistent with specific binding of DNR. Furthermore, many of these residues are not lipid-exposed but face the putative substrate chamber formed between the two TMDs. These residues are proximal to two Trps, which likely explains the previously reported quenching (29). Our results suggest DNR partitions to the membrane and then binds MsbA in a manner consistent with the hydrophobic cleaner model. Interpretation in the context of the crystal structures of MsbA identifies a putative translocation pathway through the transmembrane segment.

**EXPERIMENTAL PROCEDURES**

**Materials**—n-Dodecyl-α-d-maltoside (α-DDM) was from Anatrace Inc.; 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethiosulfonate spin label was from Toronto Research Chemicals; nickel-nitritolactate resin was from Qiagen Inc.; Superdex 200 column was from GE Healthcare; DNA oligonucleotides were from Integrated DNA Technologies (IDT); tris(2-carboxyethyl)phosphine (TCEP) was from Molecular Probes; and DNR, ATP, and AMP-PNP were from Sigma.

**Cloning and Expression of MsbA Mutants**—Site-specific mutations of MsbA within a pET19b expression vector were generated using QuikChange (Stratagene) site-directed mutagenesis and were expressed in Escherichia coli BL21(DE3) in minimal medium A using an induction temperature of 28–30 °C as described previously (22, 23).

**Purification and Labeling of MsbA Mutants**—Membranes from MsbA mutants solubilized in 1.25% w/v α-DDM and 0.5 mM dithiothreitol were purified using Ni²⁺ affinity chromatography and spin-labeled through two additions of 20-fold excess 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethiosulfonate spin label for 4 h at room temperature followed by overnight storage on ice. The labeled proteins were further purified by size-exclusion chromatography in a gel filtration buffer (50 mM HEPES, 50 mM NaCl, and 0.015% α-DDM), using a Superdex 200 column that also removed unreacted spin label.

**DNR Fluorescence Anisotropy**—The fluorescence anisotropy of DNR in buffer (50 mM HEPES, 50 mM NaCl, pH 7.2) with and without 0.05% α-DDM was measured in a spectrofluorometer (Photon Technology International) equipped with polarizers. The fluorescence intensities parallel and perpendicular to the direction of polarized light were analyzed to determine steady state anisotropy values from Equation 1,

\[
r = \frac{I_{VV} - G \cdot I_{HH}}{I_{VV} + 2G \cdot I_{HH}}
\]

where \( G \) is the instrument correction factor and is given by the ratio of \( I_{VV}/I_{HH} \). To establish the interaction of DNR with MsbA, different concentrations of detergent-solubilized cysteine-less MsbA (Wt*) (2–20 μM) were incubated with DNR (5 μM) for 15 min at 37 °C, and the change in DNR fluorescence anisotropy was monitored. The samples were excited at 485 nm, and the fluorescence signal was monitored at 555 nm with both excitation and emission slit width set to 1 nm.

**Trp Fluorescence Quenching Studies**—Trp fluorescence quenching by DNR was measured following incubation of MsbA (2 μM) or free Trp (1 μM) in buffer (50 mM HEPES, 50 mM NaCl, and 0.05% α-DDM, pH 7.2) with 0–200 μM of DNR (with and without 5 mM AMP-PNP for 30 min at 37 °C) for 15 min at 37 °C. Samples were excited at 295 nm, and emission was monitored at 330 nm for Trps within proteins and 350 nm for free Trp. The data are plotted as Stern-Volmer plots (38), where \( F_0 \)
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and F are the fluorescence intensities in the absence and presence of DNR.

**DNR Fluorescence Quenching Studies by Spin-labeled Proteins**—Site-specific spin-labeled mutants of helices 3, 4, and 6 of NBD either in the WT* or W10F/W17F background (10 \( \mu M \)) were incubated with and without 500 \( \mu M \) of AMP-PNP for 30 min followed by incubation with DNR (5 \( \mu M \)) for 15 min at 37 °C. Reversal in DNR quenching on releasing the spin label from spin-labeled proteins (10 \( \mu M \)) was measured by preincubating the samples with TCEP (100 \( \mu M \)) for 15 min at room temperature followed by incubation with DNR (5 \( \mu M \)) for 15 min at 37 °C. Change in DNR fluorescence was measured at emission wavelength of 555 nm after excitation at 485 nm.

**EPR Spectroscopy**—EPR spectra of spin-labeled proteins (100 \( \mu M \)) were collected on a Varian E-109 spectrometer fitted with a loop-gap resonator as described previously (39). To release the spin label from labeled proteins, samples (100 \( \mu M \)) were incubated with TCEP (1 \( mM \)) for 15 min at room temperature. Samples were loaded in capillaries, and spectra were collected using 2 milliwatts of microwave power with a 160-G field scan.

**ATPase Assay**—The specific ATPase activity of MsbA mutants was determined as described previously (22). Briefly, purified MsbA samples were incubated with different concentrations of ATP at 37 °C for 20 min. The reaction was stopped by adding SDS, and the color was developed using a 1:1 solution of ammonium molybdate (2% in 1 \( M \) HCl) and ascorbic acid (12% in 1 \( M \) HCl). The absorbance of samples was measured at a wavelength of 850 nm. The amount of phosphate released was determined by comparing with inorganic phosphate standards.

**RESULTS**

**MsbA Binds DNR**—To establish that DNR (Fig. 1A) specifically interacts with MsbA, we measured the change in DNR rotational diffusion in the presence of detergent-solubilized MsbA. Small fluorescent molecules weakly depolarize light because of rapid tumbling in aqueous media. Binding to MsbA is expected to slow the rate of DNR tumbling thereby increasing fluorescence anisotropy. DNR anisotropy in detergent-free buffer is negligible (0.002 ± 0.001) and increases in the presence of \( \alpha \)-DDM (0.1 ± 0.007) consistent with DNR partitioning into the detergent micelles (Fig. 1B). A further increase in anisotropy is observed with increasing MsbA concentration suggesting a direct interaction between DNR and MsbA. The binding has a dissociation constant \( (K_d) \) of 4.7 \( \mu M \), assuming one binding site per functional dimer, and saturates over a range of 15–20 \( \mu M \) MsbA.

**DNR Quenches MsbA Trp Fluorescence**—Previous studies report DNR quenches intrinsic Trp fluorescence of MsbA (29). Five MsbA Trps are distributed on different helices near the membrane/water interface (19). Trp-10 and Trp-17 are in a short so-called “elbow helix” parallel to the plane of the membrane preceding transmembrane helix 1 (TM1), Trp-66 and Trp-91 are in helix 2 (TM2), and Trp-165 is part of extracellular loop 2 (EL2) (Fig. 2A). The fluorescence emission spectrum of Trps in WT* exhibits a maximum at 330 nm consistent with the location of Trps in a hydrophobic environment.

DNR quenches MsbA Trp fluorescence without a concomitant spectral shift (data not shown). The corresponding Stern-Volmer plot (Fig. 2B) displays a pronounced curvature at DNR concentrations higher than 20 \( \mu M \), generally attributed to a mixed static-dynamic quenching mode (38). Formation of a complex with DNR in close proximity to a subset of the Trp residues likely accounts for the static term. In comparison, the Stern-Volmer plot of free Trp in solution is linear, and the quenching is consistently smaller compared with that of WT* (Fig. 2C). Quenching of free Trp exclusively arises from dynamic collisions in bulk solution.

To establish that the quenching of MsbA Trps by DNR is a consequence of complex formation, the Trp emission intensity was measured following sequential incubation with AMP-PNP and DNR. Binding of AMP-PNP, a nonhydrolyzable analog of ATP, triggers conformational rearrangements that close the substrate binding chamber to the cytoplasm (19). Models of transport predict a concomitant substrate movement to a low affinity site, which is presumably to be at the periplasmic side (7). Preincubation with AMP-PNP eliminates DNR access thereby reducing quenching as evident by the reduced Stern-Volmer slope (Fig. 2B). In contrast, addition of AMP-PNP does not affect free Trp quenching (Fig. 2C). Furthermore, the Stern-Volmer plots for AMP-PNP-bound MsbA and free Trp are superimposable consistent with a base line of dynamic collidi-
lations between MsbA and DNR (Fig. 3A). Given that AMP-PNP binding closes the cytoplasmic side of MsbA (19), these results support a model wherein DNR association occurs at this side of the transporter.

**DNR Specifically Binds Near Trp-10 and Trp-17 of the Elbow Helix**—The conclusion above implies that quenching by DNR should occur primarily at Trp-10, -17, and -91 (Fig. 2A). To test this model, we individually substituted each of these Trps (10, 17, and 91) to phenylalanines as well as generated double mutant W10F/W17F and triple mutant W10F/W17F/W91F. The fluorescence emission intensities of the double and triple mutants decrease by 30% without any change in emission maxima (data not shown). These mutant proteins are catalytically active with $V_{\text{max}}$ and $K_{m}$ values comparable with WT* ($V_{\text{max}}$ 1.2 $\mu$mol/min/mg; $K_{m}$ 0.280 mM) (supplemental Fig. 1). The W10F/W17F and W10F/W17F/W91F substitutions reduce quenching by DNR to a level similar to that of free Trp (Fig. 3A). Furthermore, this residual quenching is not sensitive to AMP-PNP, suggesting that it arises from dynamic collision between DNR and Trps at sites 66, 91, and 165.

These results map the AMP-PNP-sensitive Trp quenching, representative of DNR binding, to a region near the cytoplasmic

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**FIGURE 2.** DNR quenches MsbA Trp fluorescence. A, ribbon representation of the MsbA structure in the open state highlighting the five Trps near the membrane/water interface. B, Stern-Volmer plots of WT* quenching by DNR in the absence and presence of AMP-PNP. C, Stern-Volmer plots of free Trp quenching by DNR in the absence and presence of AMP-PNP. $F_0$ and $F$ are the fluorescence intensities in the absence and presence of DNR. Each point represents the average of more than three measurements. The error bars are the standard deviations.
elbow helix. The similar quenching pattern of double and triple mutants suggests that the emission intensity of Trp-91 is not affected by DNR binding. Analysis of the single substitutions W10F, W17F, and W91F further supports this conclusion. Stern-Volmer plots of W10F and W17F have reduced slopes compared with WT* (Fig. 3B) and exhibit smaller quenching levels in the presence of 20 μM DNR compared with WT* (Fig. 3C) in contrast to W91F, which has a quenching level comparable with WT* (Fig. 3, B and C). Slopes of Stern-Volmer plots for all the mutants are similarly reduced in the presence of AMP-PNP (Fig. 3B).

Spin-labeled Residues at the Cytoplasmic Sides of Helix 3 and 6 Quench DNR Fluorescence—DNR binding to the MsbA cytoplasmic side would involve a number of residues from different helices. To expand identification of these residues beyond the endogenous Trps, we took advantage of DNR fluorescence quenching by proximity to paramagnetic spin labels. Because Trp-10 and -17 face the hydrocarbon phase, residue Leu-143 along the lipid-facing surface of helix 3 was replaced with cysteine in the W10F/W17F background and spin-labeled as described under "Experimental Procedures." Additionally, several chamber-facing residues, including Arg-296 and Gln-307 in helix 6, Ile-144 and Thr-145 in helix 3, and Arg-183 in helix 4, were also selected for spin labeling. Finally, the NBD solvent-exposed residue Thr-561 serves as a control for bulk solution collision between spin-labeled MsbA and DNR. All spin-labeled mutants migrate on size-exclusion chromatography with retention times similar to WT and hydrolyze ATP at rates consistent with minimal perturbation by the cysteine substitution and subsequent spin labeling (supplemental Fig. S2 and supplemental Table 1). These mutants are benchmarked relative to the spin label substitution at site 482, which shows no detectable ATPase activity. The serine residue at position 482 is highly conserved among ATP transporters and is required for catalysis.

Spin labels at residues Leu-143, Ile-144, Thr-145 (helix 3), Arg-296, and Gln-307 (helix 6) but not at residues Arg-183 (helix 4) or Thr-561 (NBD) reduce DNR fluorescence indicating the close proximity is required for quenching through direct collision of the spin labels with DNR (Fig. 4A). Furthermore, addition of AMP-PNP to trap the transporter in an outward-facing conformation eliminates the quenching by spin labels. Given that residues Thr-145, Arg-296, and Gln-307 face the chamber in the open conformation and the sensitivity of quenching to AMP-PNP, collision between spin labels and bulk DNR in detergent micelles cannot be responsible for the observed quenching.

Fig. 4B shows the change in DNR emission intensity upon titration with increasing concentrations of MsbA spin-labeled at the chamber-facing site Ile-144. The resulting curve has a shape characteristic of saturable binding, whereas those for Arg-183 and W10F/W17F are flat (Fig. 4C). The $K_D$ value obtained from the Ile-144 (4.8 μM) curve is similar quenching of Trp fluorescence by 20 μM DNR in the absence and presence of AMP-PNP. $F_0$ and $F$ are the fluorescence intensities in the absence and presence of DNR. Each point represents the average of more than three measurements. The error bars are the standard deviations.

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)
to that obtained from analysis of the anisotropy data in Fig. 1B. Together, these results demonstrate that the interaction between DNR and spin-labeled residue Ile-144 is affinity-mediated.

Releasing spin label by the addition of TCEP eliminates DNR quenching further demonstrating the quenching specificity (Fig. 5A). Free, unattached spin labels give rise to a characteristic highly mobile spectrum dominated by sharp, narrow features (Fig. 5B). The amplitude of high field signal directly reports the fraction of released spin label. Analysis of the
change in the EPR line shape upon incubation with TCEP confirms that at least 60% of spin label was released at most sites (supplemental Table 2).

A more extensive screen for residues in proximity to bound DNR was carried out using spin-labeled mutants in the WT* background. Unlike W10F/W17F, binding of DNR to unlabeled WT* results in AMP-PNP-sensitive quenching. However, the relative spin label contribution can be easily separated by comparing fluorescence levels before and after spin label release by TCEP. Spin-labeled residues 142–148 and Gly-150 of helix 3; Arg-296, Ser-300, Asn-303, and Gln-307 of helix 6; and Ile-182 and Arg-190 of helix 4 quenched DNR fluorescence by 30–35% indicating proximity to DNR (Fig. 6, A and B). DNR recovers full emission intensity when MsbA is trapped in a closed conformation by addition of AMP-PNP (Fig. 6, A and B) or if the spin label is released by TCEP (Fig. 6C). In comparison, WT* quenches DNR fluorescence to about 15%, which can be attributed to Trp-10 and -17 (Fig. 4A and Fig. 6A). Spin-labeled mutants Glu-149 and 151–162 of helix 3 and most of helix 4 residues did not quench DNR fluorescence above the WT* level.

Although the analysis above establishes that specific binding to MsbA causes DNR quenching by spin labels, the absence of quenching at some sites may be influenced by variations in the reactivity of the spin labels. Therefore, we determined the relative labeling efficiency of the various MsbA mutants by comparing the amplitude of doubly integrated EPR spectra at the same protein concentration (representative sites reported in supplemental Table 2). As expected, lipid-exposed residues near the middle of the bilayer were labeled at lower efficiencies. Nevertheless, no correlation was observed between labeling efficiency and DNR quenching. For instance, the lipid facing Gly-150 in the middle of helix 3 is one of the least labeled sites (~20–30%), yet it clearly demonstrates AMP-PNP-sensitive quenching. In contrast, the spin label at site Arg-188 of helix 4 does not reduce DNR emission intensity beyond the background despite almost stoichiometric labeling. The fact that spin labels at residues along helix 4 do not quench DNR suggests that labeling efficiency is not the sole determinant of fluorescence change. However, variations in labeling intensity confound quantitative quenching analysis prompting a binary classification of residues.

In contrast to DNR quenching by spin labels in the TMD, a survey of selected residues in the NBDs shows minimal effects on DNR emission intensity. Spin-labeled residues Glu-343, Leu-539, and Thr-561 did not quench DNR fluorescence above the WT* level (Fig. 6B). Because these are the residues most exposed to the aqueous phase, the lack of DNR quenching at NBD sites supports our conclusion that DNR specifically interacts with the TMD of MsbA.

**DISCUSSION**

This work reports the first detailed mapping of substrate-binding sites in the transmembrane domain of MsbA. A previous study from our laboratory demonstrates that LPS binding leads to a uniform increase in distances across all three MsbA domains (23). Given their size difference, DNR and LPS likely interact with MsbA through different modes with the former

![Figure 6](https://example.com/figure6.png)
binding in a more localized area. Previously, Eckford and Sharam (29) demonstrated that DNR binding affects the emission intensity of the Trp residues of MsbA. Here we assign these intensity changes to two Trps in the elbow helix at the membrane/water interface. We also identify additional residues in the DNR binding region through selective DNR quenching by spin labels covalently attached to MsbA (37). This approach can be applied more generally to other lipophilic substrate molecules whose hydrocarbon phase partitioning confounds biochemical analysis. The resolution of this technique is limited by the length of the spin label linking arm, which has the effect of increasing the apparent number of residues in proximity to the substrate.

Although the residues where spin labels quench DNR fluorescence reside on different helices, they still cluster together in crystal structures of MsbA. In the open structure (Fig. 7), some of these residues face the lipid bilayer just below the elbow helix, whereas others face the chamber. Polar residues of helices 3 (Thr-145 and Arg-148) and 6 (Arg-296, Ser-300, Asn-303, and Gln-307) reporting proximity to DNR are stacked facing the open chamber. The remaining hydrophobic helix 3 residues (Ala-142, Leu-143, Val-146, Val-147, and Gly-150) face away from the chamber either toward neighboring helices or the membrane. In the context of the AMP-PMP-bound outward-facing conformation (19), chamber-facing residues are shielded consistent with DNR exclusion and concomitant elimination of spin label quenching in the presence of AMP-PNP (Fig. 8). The fact that these residues identified through the quenching screen line up with the outward-facing chamber in the crystal structure (19) suggests a translocation pathway through the TMD. The lack of quenching by most spin labels introduced along helix 4 is consistent with its peripheral location in both structures. This result provides further evidence that the quenching observed arises from formation of an MsbA-DNR complex rather than from dynamic collisions within micelles. The sensitivity of fluorescence of Trp-10 and -17 to DNR binding can either be due to direct proximity to the binding site or to
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DNR-induced conformational changes that alter the local environment of Trps. Comparison of the two MsbA structures reveals little change in the elbow helix environment. Thus, the loss of Trp quenching upon ATP binding likely reflects the exclusion of DNR from the binding pathway rather than propagated structural changes.

The simplest model consistent with our data describes DNR binding the open conformation just below the elbow helix becoming sandwiched in the 7.9 Å space between Trp-10 and -17. This interaction is reminiscent of the substrate pocket of riboflavin-binding protein in which the ligand riboflavin forms a compact π stack between Trp-156 and Tyr-75 with equal interplanar distances of 3.7 Å (40, 41). DNR and riboflavin have similar planar three-ring anthraquinone-type structures, and it has been suggested that DNR inserts in the riboflavin-binding protein site (42). The distribution of the spin label quenching sites, particularly along helix 3, reflects a diffuse location of bound DNR. We suggest that from the binding site near the elbow helix, DNR translocates to the cytoplasmic side of the open channel where it contacts channel-facing residues in helices 3 and 6.

The notion of distinct substrate entrance and binding locations has been invoked to describe binding of the multidrug transporter AcrB to a variety of substrates, including doxorubicin, an analog of DNR (43). Substrates enter an opening at the membrane-periplasm boundary, pass through a binding pocket, and are extruded through a funnel that connects to the TolC channel (44). Access to the binding site is controlled by a vestibule near the water/membrane interface (43). The voluminous binding site uses aromatic residues to recognize a wide variety of substrates, a motif previously observed in the transcriptional regulator QacR that binds multiple drugs (45, 46). Doxorubicin interacts with a cluster of phenylalanine and glutamine residues in the AcrB-binding site (43). Although our analysis is resolution-limited, the hydrophobic nature of most residues in proximity to DNR is noteworthy. Neighboring phenylalanine and glutamine on the elbow helix of MsbA together with the hydrophobic residues facing the bilayer suggest DNR binding to the region enclosed by helices 1, 3, and 6 is plausible.

In summary, DNR partitions into the inner leaflet of the inner membrane and binds between Trp-10 and -17 in the interfacial region. An interfacial binding site has also been proposed for ABCB1 substrates H33342 and LDS-751 (47, 48). This model is consistent with the hydrophobic cleaner model wherein multidrug resistance transporters pick up their amphipathic substrates from the membrane rather than the aqueous phase. DNR then enters the funnel-shaped area bound by helices 3 and 6 (Fig. 7). In the ATP (AMP-PNP)-bound intermediate, the MsbA chamber undergoes a conformational change from inward- to outward-facing, thereby extruding DNR to the periplasm or the outer leaflet of the lipid bilayer along the lines of an alternating access model (Fig. 8). Using a similar fluorescence quenching by spin labels approach, it should be possible to test this model and further define the regions of the DNR periplasmic binding site.

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