Functional Characterization of *Escherichia coli* MsbA

**INTERACTION WITH NUCLEOTIDES AND SUBSTRATES**

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The *Escherichia coli* MsbA protein is a 65-kDa member of the ATP-binding cassette superfamily. It is thought to function as an ATP-dependent lipid translocate that transports lipid A from the inner to the outer leaflet of the cytoplasmic membrane. MsbA with high ATPase activity was isolated and found to be homodimeric in detergent solution. The protein ATPase activity was inhibited by vanadate and showed variable patterns of stimulation and inhibition by lipid A and other compounds. The intrinsic tryptophan fluorescence of the protein was characterized, and dynamic quenching using acrylamide showed that a conformational change took place on binding of lipid A. Fluorescence quenching was used to characterize the interactions of MsbA with nucleotides and various putative substrates, including lipids, lipid-like compounds, and drugs. MsbA had an apparent binding affinity for ATP of ~2 mM and also bound nonhydrolyzable ATP analogs and fluorescent ATP derivatives. The putative substrate lipid A interacted with the protein with an affinity of 6.4 μM. Drugs that are known to be substrates for ABC multidrug transporters also interacted with MsbA with affinities in the range 0.25–50 μM. This study represents the first use of fluorescence approaches to estimate MsbA binding affinities for nucleotides and putative transport substrates.

The outer membrane of Gram-negative bacteria possesses a unique asymmetric structure. The inner leaflet is composed primarily of glycerophospholipids, although with a higher proportion of phosphatidylethanolamine (PE) and saturated acyl chains than the cytoplasmic membrane (reviewed in Ref. 1). The outer leaflet of the outer membrane is composed mainly of lipid A (Fig. 1), the hexa-acylated hydrophobic core lipid of lipopolysaccharide (LPS) (reviewed in Ref. 2). Our understanding of how these lipids traffic to the outer membrane, and how lipid asymmetry is maintained, is limited. One important component of this lipid trafficking system appears to be MsbA (Fig. 1), an essential 64.5-kDa protein found in the cytoplasmic membrane. MsbA is a member of the ATP-binding cassette (ABC) superfamily of proteins, and is proposed to translocate the LPS precursor lipid A (and possibly also phospholipids) from the inner to the outer leaflet of the cytoplasmic membrane. MsbA-mediated translocation is thought to be the first step in the movement of lipid A from its site of synthesis on the inner surface of the cytoplasmic membrane to its final location in the outer leaflet of the outer membrane.

MsbA is a 582-residue integral protein with six putative transmembrane (TM) helices and one ABC-type nucleotide-binding (NB) domain at its C-terminal end (Fig. 1). It is presumed to function as a dimer. The sequence of MsbA is 30% identical and 46% similar to that of the N-terminal half of P-glycoprotein (Pgp; ABCB1; MDR1) (3), a mammalian efflux pump implicated in multidrug resistance to anti-cancer agents (reviewed in Ref. 4). MsbA is a further 51% identical and 66% similar to Pgp in the NB regions (3), and it is one of the prokaryotic proteins most homologous to a mammalian ABC protein.

The x-ray crystal structures of MsbA from three bacterial species in different conformations were recently reported (5), after the original MsbA structures were withdrawn due to the discovery of a flaw in the software used to solve them (6). A new AMP-PNP-bound structure of MsbA was solved to 3.7 Å, and showed a series of interacting helices that span the bilayer and extend from the membrane into the cytosol, where they are coupled to two interdigitated NB domains (5). The overall shape and domain organization of MsbA resemble that of the 3.0 Å structure of the putative bacterial multidrug transporter Sav1866 (7) and the 8 Å cryo-EM structure of Pgp (8).

The ATPase activity of MsbA reconstituted into *Escherichia coli* lipids could be stimulated 4–5-fold in the presence of 3-deoxy-d-manno-2-octulosonic acid-lipid A (9), demonstrating that MsbA is a lipid-activated ATPase. Reuter et al. (10) reported that MsbA and LmrA (a bacterial homolog of Pgp) had similar substrate specificities. MsbA overexpression in *E. coli* conferred resistance to ethidium bromide, by apparent energy-dependent transport of the molecule from intact cells. The protein could be photolabeled by the LmrA/Pgp photoactive substrate [1H]azidopine, and like LmrA and Pgp, its ATPase activity in membrane vesicles was stimulated by daunorubicin, vinblastine, and Hoechst H33342 (H33342). Finally, ATP-dependent transport of H33342 could be detected in proteoliposomes containing MsbA. A more recent study reported that...
transport of ethidium bromide and H33342 by MsbA overexpressed in *Lactococcus lactis* was inhibited by lipid A (11).

Several lines of evidence suggest a physiological role for MsbA in the translocation of lipid A, and possibly phospholipids, from the inner to the outer leaflet of the cytoplasmic membrane. Mutations in MsbA result in accumulation of both lipid A derivatives and phospholipids in the inner membrane of *E. coli* (12). In *E. coli* MsbA missense mutants at the nonpermissive temperature, both PE and lipid A were accessible to chemical and enzymatic modification from the cytoplasmic rather than the periplasmic face (13), consistent with a model of MsbA-mediated translocation between membrane leaflets, rather than ejection from the bilayer. Several ABC proteins translocate or “flip” phospholipids from the inner to the outer leaflet of the membrane, suggesting that MsbA may function in the same way. For example, LmrA, Pgp, and MRP1 can mediate flip-flop of fluorescently labeled lipids in a reconstituted system (14–17).

We report here the purification of MsbA with very high levels of ATPase activity (0.9–1.0 μmol of ATP hydrolyzed per min/mg of protein) in both detergent solution (where it appears to exist as a dimer) and reconstituted proteoliposomes. MsbA ATPase activity was modulated by lipid A, lipid-like compounds, and other molecules known to be substrates for ABC multidrug transporters. This work characterizes the intrinsic Trp fluorescence of purified functional MsbA for the first time and examines possible conformational changes arising from nucleotide and substrate binding. Fluorescence quenching studies are used to demonstrate the interaction of nucleotides, lipids, and related molecules with the protein and quantify their binding affinity. Such studies are a necessary first step to understanding the transport cycle and catalytic mechanism of this protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—*N*-Dodecyl-β-d-maltoside (DM) was obtained through Alexis Biochemicals (San Diego, CA). Nickel-nitrilotriacetic acid–agarose resin was purchased from Qiagen (Mississauga, Ontario, Canada). Bio-Beads SM-2 adsorbent was purchased from Bio-Rad. ADP, ATP, AMP-PCP, AMP-PNP, lipid A, RaLPS, ReLPS, colchicine, daunorubicin, dithioerythritol (DTE), sodium orthovanadate, verapamil, vinblastine and *N*-acetyl-1-tryptophanamide (NATA), were purchased from Sigma. *E. coli* lipid A were purchased from Avanti Polar Lipids (Alabaster, AL). H33342, TNP-ATP, and TNP-ADP were obtained from Molecular Probes (Eugene, OR). Roche Diagnostics generously provided ilmofosine (1-β-hexadecylthio-2-methoxy-methyl-2-deoxy-rac-glycero-3-phosphocholine). D-20133 (octadecyl-(2-(N-methyl-piperidino)-ethyl)-phosphate) and D-21266 (octadecyl-(N,N-dimethyl-piperidino-4-yl)-phosphate) were gifts from ASTA Medica (Toronto, Ontario, Canada). Edelfosine (1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine) was obtained from Calbiochem. Lipid A, the rough chemotype RaLPS, and the smooth chemotype ReLPS were prepared as stock solutions in the appropriate buffer. Other drugs were prepared as stock solutions in DMSO. *E. coli* lipid stocks were prepared in 4:1 (v/v) chloroform/methanol and stored at −20 °C.

**MsbA Expression and Purification**—A plasmid containing wild-type, N-terminally His6-tagged *E. coli* MsbA was provided by Dr. Candice Klug of the Medical College of Wisconsin (18). The protein was overexpressed in *E. coli*, essentially as described previously (18). Briefly, the plasmid was transformed into BL21 DE3 cells for expression, and the cells were grown in 1-liter flasks in the presence of 30 μg/ml kanamycin A at 37 °C. Cells were induced with 1 mM isopropyl 1-thio-β-d-galactopyranoside for 3 h before harvesting. Inside-out membrane vesicles were prepared by cell lysis using a French press at a pres-
sure of 10,000 p.s.i., followed by both low and high speed centrifugation of the cell lysis mixture. The vesicles were lysed with 1% (w/v) DM, and MsbA was purified using a nickel-nitritotriacetic acid column equilibrated with buffer containing 0.1% (w/v) DM. Protein was assayed by the method of Peterson (19) using a bovine serum albumin standard. SDS-PAGE was carried out according to Laemmli (20), using a 10% acrylamide gel with 50 μg of membrane protein or 10 μg of purified MsbA in 0.1% (w/v) DM, followed by staining with Coomassie Blue. An unstained gel was transferred to a nitrocellulose membrane and probed with mouse anti-His\textsubscript{6} antibody (Qiagen, Mississauga, Ontario, Canada), followed by goat anti-mouse IgG-horseradish peroxidase conjugate. Samples were detected using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Biosciences).

**Determination of the Oligomeric State of MsbA by Gel Filtration Chromatography**—Gel filtration sizing analysis was carried out on a 10 × 300 mm Superose 610/300 GL column (Amer sham Biosciences) using an AKTA fast protein liquid chromatography system. After column equilibration with 20 mM HEPES buffer containing 150 mM NaCl, 5 mM MgCl\textsubscript{2}, and 0.05% (w/v) DM, pH 7.5, a 100-μl sample (1.7 mg/ml) of purified MsbA or protein standard (aldolase, 158 kDa; transferrin, 81 kDa; or ovalbumin, 45 kDa) was applied to the column. Proteins were eluted at 0.5 ml/min, and elution was monitored by absorbance at 280 nm. The molecular weight of MsbA was interpolated by comparison of its elution volume to those of the protein standards.

**Reconstitution of MsbA into Proteoliposomes**—MsbA was reconstituted into proteoliposomes of *E. coli* lipids by mixing 5 mg of dried lipid with 500 μl of 1% (w/v) DM and 1 ml of purified MsbA at the desired concentration in reconstitution buffer (50 mM HEPES, 100 mM KCl, 5 mM MgCl\textsubscript{2}, 2 mM DTE, pH 7.5). After solubilization, three additions were made of 200 mg each of Bio-Beads SM-2 adsorbent, with stirring for 3 h on ice. The sample was removed from the Bio-Beads using a fine needle and centrifuged for 20 min at 37,000 × g, and the proteoliposome pellet was resuspended in fresh buffer. Proteoliposome size measurements were carried out using a Zetasizer Nano (Malvern Instruments, Southborough, MA).

**ATPase Activity Measurements**—ATPase activity associated with inside-out membrane vesicles, purified MsbA in DM, and MsbA reconstituted into proteoliposomes was determined by the method of Chifflet et al. (21) as described previously (22), but using 20 mM HEPES, 100 mM NaCl, 2 mM DTE, 5 mM MgCl\textsubscript{2}, pH 7.5, as the ATPase buffer and a final concentration of 5 mM ATP.

**Measurement of Substrate Binding Affinity by Intrinsic Fluorescence Quenching**—Binding affinity was determined for a variety of nucleotides, lipids, lipid-like compounds, and other molecules using quenching of the intrinsic Trp fluorescence of purified MsbA. The protein was solubilized in 0.1% (w/v) DM at a concentration of 100 μg/ml in ATPase buffer. Fluorescence measurements were carried out as described previously (22, 23) using a PTI QuantaMaster C-61 steady-state fluorimeter (Photon Technology International, London, Ontario, Canada), with a 2 nm bandwidth for both excitation (λ\textsubscript{ex} = 290 nm) and emission (λ\textsubscript{em} = 330 nm). Nucleotides, lipid A, RaLPS, and ReLPS were added as solutions in ATPase buffer, and other compounds were added as DMSO solutions. Data were collected after equilibration at 20 °C for drugs and lipids or 10 °C for nucleotides (to limit hydrolysis by the protein). Fluorescence data were corrected for inner filter effect, dilution, and scattering, where appropriate (25), and fitted to Equation 1, which describes binding to a single site.

\[ \frac{\Delta F}{F_0} = \frac{\Delta F_{\text{max}}}{F_0} \times \frac{100}{K_d + [S]} \]  

(Eq. 1)

where (\(\Delta F/F_0 \times 100\)) is the percent fluorescence quenching (percent change in fluorescence relative to the initial value, \(F_0\)), following addition of substrate at a concentration \([S]\), and \(K_d\) is the dissociation constant for binding to the protein. Fitting was carried out using nonlinear regression (SigmaPlot, SPSS Inc., Chicago), and values of \(K_d\) and the maximum percent fluorescence quenching, (\(\Delta F_{\text{max}}/F_0 \times 100\)), were extracted. Control titrations were performed with 30 μM NATA to assess the non-specific quenching of Trp fluorescence by nucleotides, lipids, and drugs.

**Fluorescence Quenching of MsbA by Acrylamide**—A stock solution of freshly prepared 5 μM acrylamide in ATPase buffer was added in 2-μl aliquots to 250 μl of 100 μg/ml purified MsbA in ATPase buffer with 0.1% (w/v) DM, in the presence or absence of 2 mM ATP and 11 μM lipid A or 10 μM H33342. Fluorescence emission was measured at 330 nm following excitation at 290 nm (slit widths of 2 nm). All data were collected after incubation at 10 °C to limit hydrolysis of ATP, where present, and to allow comparison between samples. Fluorescence intensities were corrected for inner filter effect, dilution, and scattering. Parallel experiments were carried out using NATA to assess acrylamide quenching of the fluorescence of Trp residues accessible in aqueous solution. Quenching data were analyzed using the Stern-Volmer Equation 2,

\[ \frac{F_0}{F} = 1 + K_{SV}[Q] \]  

(Eq. 2)

where \(F_0\) and \(F\) are the fluorescence intensities in the absence and presence of acrylamide, respectively; \([Q]\) is the concentration of acrylamide, and \(K_{SV}\) is the Stern-Volmer quenching constant. For a collisional quenching mechanism, a plot of \(F_0/F\) versus \([Q]\) gives a straight line with a slope of \(K_{SV}\).

**RESULTS**

**Isolation and Purification of MsbA**—N-terminally His\textsubscript{6}-tagged *E. coli* MsbA was overexpressed in *E. coli* BL21 DE3 cells and purified by nickel-chelate chromatography. Isolated bacterial membrane vesicles contained roughly 5–10% MsbA as assessed by Coomassie Blue-stained SDS-PAGE, and purified protein in detergent was >90% pure (Fig. 2). A typical preparation from 2 liters of bacterial culture yielded 160 mg of membrane protein with ATPase-specific activity of ~100 nmol/min per mg of protein, and 4.5–5.0 mg of purified MsbA in detergent solution with specific activity of ~0.9–1.0 μmol/min per mg of protein. Maximal ATPase activity was reached at ATP concentrations greater than 5 mM and 5 mM MgCl\textsubscript{2}. A 20-min
assay time was used, because ATP hydrolysis was still linear at times beyond this period. The addition of DTE to the buffers at a concentration of 2 mM increased the measured ATPase activity of the protein 5-fold compared with the value in its absence.

The oligomeric state of MsbA in DM solution was assessed using gel filtration fast protein liquid chromatography. Results showed that when compared with protein standards of known size, the elution volume of the MsbA peak corresponded to an estimated molecular mass of $145 \text{ kDa}$ (Fig. 3). Although bound DM will contribute to the protein size (pure DM micelles have a molecular mass of $50 \text{ kDa}$), it seems likely that MsbA exists as a dimer under these conditions. Traces of a band with molecular weight corresponding to the MsbA dimer could also be observed on SDS-PAGE of the purified protein (Fig. 2). Because dimerization of the NB domains is necessary for catalysis in ABC proteins, it is likely that monomeric MsbA would have very low ATPase activity.

MsbA was reconstituted into proteoliposomes of *E. coli* lipids using DM to solubilize the lipid and MsbA, and BioBeads for detergent removal. Proteoliposome size measurements made using dynamic light scattering indicated that *E. coli* lipid-MsbA proteoliposomes prepared using this method were large, with a mean diameter of around $1.8 \mu \text{m}$ (data not shown). The size profile of the proteoliposomes was monodisperse and reproducible. Aggregation of smaller proteoliposomes cannot be ruled out, but a broad size distribution would be expected in this case. Reconstitution lowered the activity of the protein by $3$-fold. Permeabilization of the proteoliposomes by the detergent CHAPS increased the ATPase activity by $50\%$, suggesting that $7/8$ of the MsbA molecules are reconstituted with their NB domains on the outer surface of largely unilamellar structures.

**Effect of Lipids and Other Compounds on the ATPase Activity of MsbA**—The ATPase activity of many ABC proteins can be stimulated and/or inhibited by their substrates. The specific activity of MsbA in DM buffer was stimulated 1.4-fold by egg PE and up to 2-fold by a mixture of *E. coli* lipids (data not shown), which contains PE, phosphatidylglycerol, and phosphatidylserine, and smaller amounts of other species such as lipid A. Addition of lipid A to purified MsbA stimulated ATPase activity more than 2-fold and did not inhibit the activity of the protein up to the highest concentration that could be easily tested, whereas RaLPS and ReLPS stimulated the activity by a more modest 20% at low concentrations and inhibited the activity at higher concentrations (Fig. 4; Table 1). The ATPase activity of MsbA was also modulated by some drugs that are substrates for drug efflux pumps such as LmrA and Pgp (Fig. 5; Table 1). Four different patterns of modulation were evident. Although drugs such as colchicine and daunorubicin neither stimulated nor inhibited the activity of MsbA in the range normally examined

*Figure 2. Purification of His$_6$-MsbA from BL21 DE3 cells overexpressing the protein.* The two left lanes show Coomassie Blue-stained 10% acrylamide SDS-PAGE of MsbA-expressing membranes (M, 50 $\mu\text{g}$ of protein) and purified MsbA in DM solution (P, 10 $\mu\text{g}$ of protein). The two right lanes show the same protein samples after Western blotting. Protein samples were transferred to a nitrocellulose membrane and probed with a mouse anti-His$_6$ antibody, followed by a goat anti-mouse IgG-horseradish peroxidase conjugate. The positions of molecular mass markers are indicated (kDa).

*Figure 3. Oligomeric state of MsbA in detergent solution.* A, MsbA was subjected to gel filtration chromatography using a Superose 610/300 GL column, and B, its elution volume was compared with that of the protein standards aldolase (ALD, 158 kDa), transferrin (TRF, 81 kDa), and ovalbumin (OVA, 45 kDa) run under the same conditions. Purified MsbA eluted with an approximate molecular mass of 145 kDa. The molecular mass of the MsbA monomer is $64.5 \text{ kDa}$.
TABLE 1

Effect of bacterial lipids, lipid-like compounds and drugs on MsbA ATPase activity

| Compound          | Concentration | SC<sub>50</sub><sup>a</sup> | Stimulation<sup>b</sup> | IC<sub>50</sub><sup>c</sup> | Inhibition<sup>d</sup> |
|-------------------|---------------|-----------------------------|-------------------------|--------------------------|------------------------|
| **Bacterial lipids** |               |                             |                         |                          |                        |
| Lipid A           |               | 65<sup>e</sup>              | >120                    |                          |                        |
| RaLPS             |               | 0.23<sup>e</sup>            | 20                      | 48<sup>e</sup>           | 87                     |
| RelPS             |               | 0.35<sup>e</sup>            | 21                      | 50<sup>e</sup>           | 82                     |
| **Lipid-like compounds** |           |                             |                         |                          |                        |
| D-21266           | —             | 51                          | 94                      |                          |                        |
| Ilmofosine        | 12            | 27                          | 115                     | >90                      |                        |
| **Drugs**         |               |                             |                         |                          |                        |
| Colchicine        | —             | —                           | —                       | —                        | —                      |
| Daunorubicin      | —             | —                           | —                       | —                        | —                      |
| H33342            | 7.5           | 179                         | >1000                   | >25                      | —                      |
| Leupeptin         | —             | —                           | 480                     | >55                      | —                      |
| LY335979          | —             | >1000                       | >49                     | —                        | —                      |
| Verapamil         | 100           | 73                          | >500                    | >34                      | —                      |
| Vinblastine       | —             | —                           | —                       | —                        | —                      |

<sup>a</sup> Concentration at which ATPase activity is 50% of the maximal observed stimulation is shown.

<sup>b</sup> Maximal stimulation of ATPase activity by the compound is shown.

<sup>c</sup> Concentration at which ATPase activity is inhibited by 50% is shown.

<sup>d</sup> Maximal inhibition of ATPase activity by the compound is shown.

<sup>e</sup> Concentration is given in micrograms/ml.

<sup>f</sup> — indicates no observed stimulation or inhibition.

for Pgp (Fig. 5A), compounds such as H33342 (Fig. 5B) stimulated activity at low concentrations and inhibited it at higher concentrations. The lipid-based drugs D-20133 (Fig. 5C) and D-21266, along with LY335979 and leupeptin (Table 1), inhibited ATPase activity but did not stimulate it at lower concentrations. Finally, verapamil displayed stimulation but no inhibition of MsbA ATPase activity up to a concentration of 0.5 mM (Fig. 5D; Table 1). We observed only modest inhibition of ~28% of the initial activity at a very high concentration of 8.1 mM.

**Effect of Trapping Agents on the ATPase Activity of MsbA—**

Vanadate (V<sub>v</sub>) is a pentavalent phosphate analog frequently employed to trap ABC proteins (and other ATPases) in a conformation that is thought to resemble the catalytic transition state. After one round of ATP hydrolysis, V<sub>v</sub> displaces Pi from the active site, trapping ADP to form a stable complex that displays no ATPase activity. Membrane vesicles isolated from E. coli cells overexpressing MsbA displayed inhibition of ATPase activity when treated with increasing concentrations of V<sub>v</sub> (Fig. 6A). The residual activity of 30–35% at high concentrations of V<sub>v</sub> may represent the catalytic activity of other ATPases in the E. coli membrane that are not sensitive to V<sub>v</sub> inhibition. Purified MsbA reconstituted into proteoliposomes of E. coli lipids also displayed inhibition by V<sub>v</sub> at similar concentrations, suggesting that most of the ATPase activity observed in membranes may be attributable to MsbA (Fig. 6B). The residual activity present at high V<sub>v</sub> concentrations is lower for reconstituted MsbA than E. coli membranes, and thus a portion (10–15%) of the residual activity in membranes may be attributed to V<sub>v</sub>-insensitive contaminating ATPases, whereas the remainder may be an intrinsic property of the protein. The V<sub>v</sub> concentration causing half-maximal inhibition of reconstituted MsbA ATPase activity was ~15 µM. The presence of the divalent cation Co<sup>2+</sup> in the buffer supported the hydrolysis of ATP by MsbA in the absence of Mg<sup>2+</sup>, with an optimal concentration of 2.5 mM, but with lower specific activity (data not shown). Aluminum fluoride (AlF<sub>3</sub>) and beryllium fluoride (BeF<sub>3</sub>) trap Pgp in similar complexes thought to resemble the transition state and ground state of the protein, respectively (26). Both of these reagents also inhibited MsbA ATPase activity (data not shown).

**Intrinsic Fluorescence of the MsbA Protein—**

The MsbA monomer includes five Trp residues near the N-terminal end of the protein (Fig. 1). In the x-ray crystal structure of AMP-PNP-bound MsbA, these residues are located close to the end of the TM helix boundaries (5). It is reasonable to assume that the Trp residues are in fact located in this region, because aromatic residues commonly cluster in the membrane interfacial zone. We examined the intrinsic Trp fluorescence of MsbA in detergent solution, with excitation at 290 nm to avoid emission from the 13 Tyr residues present in each monomer (Fig. 7). The observed emission maximum of 325 nm was highly blue-shifted compared with the water-soluble Trp analog, NATA (348 nm), which suggests that the Trp residues contributing to fluorescence emission reside in a nonpolar environment. Denaturation of MsbA with 6 M guanidine hydrochloride (GdnHCl) broadened the spectrum, lowered the fluorescence intensity, and caused a large red shift in the emission maximum, which approached that of NATA. Subsequent boiling of the sample red-shifted the emission maximum even further.

**Quenching of MsbA Trp Residues by Acrylamide—**

Acrylamide is often employed as a dynamic quencher of Trp fluorescence. Acrylamide is polar but uncharged and shows minimal penetration into the tightly packed protein interior. Thus acrylamide quenching provides an indication of the solvent exposure of Trp residues. Acrylamide was an excellent quencher of NATA fluorescence but quenched the fluorescence of MsbA Trp residues much less effectively. The Stern-Volmer plot for MsbA was linear, indicating that a single class of Trp residues is quenched, and all are equally accessible to acrylamide. The value of K<sub>SV</sub> for NATA was ~6.4-fold higher than the value for MsbA (Table 2), which indicates that the Trp residues contrib-
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utting to MsbA intrinsic fluorescence are buried or otherwise inaccessible to solvent. In the presence of ATP, lipid A, or both lipid A and ATP, acrylamide quenching also produced linear Stern-Volmer plots. The $K_{SV}$ for acrylamide quenching changed slightly (~7%) in the presence of ATP, whereas addition of lipid A decreased the $K_{SV}$ value by ~20%, indicating that the Trp residues become less accessible to solvent in the presence of substrate. Addition of both ATP and lipid A decreased the $K_{SV}$ value by roughly the same amount as addition of lipid A alone. Thus there appears to be a small conformational shift upon binding of substrate, whereas ATP binding in the absence or presence of lipid A has a much lower effect on Trp solvent accessibility. Addition of the MDR substrate H33342 decreased the Trp accessibility to a greater extent than lipid A (and quenched the Trp fluorescence to a greater extent than lipid A, see below), but it also showed a similar effect in the absence or presence of ATP (Table 2).

Quenching of MsbA Trp Fluorescence by Nucleotides, Lipids, and Drugs—Because MsbA is an ATPase, we hypothesized that binding of nucleotides to its NB domains may quench the intrinsic Trp fluorescence. As shown in Fig. 8, ATP quenched MsbA fluorescence in a saturable manner. By fitting the quenching data to an equation describing the interaction of the nucleotide with a single type of binding site on the protein (solid lines in Fig. 8), we estimated the dissociation constant for binding, $K_d$, and the maximal quenching reached upon saturation, $\Delta F_{\text{max}}$ (Table 3). ATP bound to MsbA with a $K_d$ value of ~2.1 mM. This value reflects ATP binding rather than hydrolysis, because quenching was measured at 10 °C where turnover is low (~5% of the activity at 37 °C). ADP (measured in the absence of added P$_i$) also bound saturably to MsbA, with similar affinity to ATP (Fig. 8; Table 3). The nonhydrolyzable analog AMP-PNP yielded a quenching curve with similar affinity and maximal quenching. The nonhydrolyzable ATP analog AMP-PCP appears to bind to MsbA with significantly higher affinity than ATP (Table 3). The fluorescent nucleotides TNP-ATP and TNP-ADP also bound to MsbA with higher affinity, with maximal fluorescence quenching of over 80% (Table 3). It is likely that quenching by these analogs takes place by Forster resonance energy transfer (FRET) between the Trp residues and the highly fluorescent TNP group following binding to the catalytic site.

Like Pgp, MsbA is able to hydrolyze TNP-ATP, albeit at a lower rate than its corresponding ATPase activity. Optimal TNP-ATP hydrolysis activity was 20% of the value obtained for 5 mM ATP and 125 mM Pi also bound saturably to MsbA, 2.5-fold higher than the $K_d$ value for binding. The presence of 5 mM AMP-PCP inhibited MsbA ATPase activity by 98%. The $IC_{50}$ value for inhibition of ATPase activity by AMP-PCP was ~1.25 mM, 4-fold greater than the $K_d$ value for binding to the protein (data not shown). These observations suggest that the tighter binding nucleotides, TNP-ATP and AMP-PCP, indeed interact with the nucleotide-binding site in the same way as ATP, rather than by an alternative mode of binding.

We anticipated that MsbA Trp fluorescence might also display quenching upon binding of substrates. Addition of increasing amounts of lipid A to purified MsbA in DM resulted in a saturable concentration-dependent quenching of the intrinsic fluorescence (Fig. 9A). As shown in Table 3, lipid A appears to bind to the protein with an affinity of 6.4 μM. Addition of RaLPS and ReLPS also led to saturable quenching of MsbA fluorescence (Fig. 9, B and C), with binding affinities of 5.9 and 8.1 μg/ml, respectively (Table 3). Some drugs modulated MsbA ATPase activity (Table 1) and thus may be substrates for the protein. We evaluated whether a variety of lipid-based compounds and MDR-spectrum drugs bound to MsbA, as assessed by intrinsic Trp fluorescence quenching (Fig. 10). We observed a range of $K_d$ values for the various compounds of over 2 orders of magnitude, from 0.23 μM to greater than 50 μM. Significantly higher maximal quenching of Trp fluorescence occurred for fluorescent compounds such as daunorubicin (~95%) and
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![Graph A](image)

**FIGURE 6.** Vanadate inhibition of MsbA ATPase activity. Increasing concentrations of vanadate were added to isolated MsbA-expressing \( E. \) coli membranes (A), and purified MsbA reconstituted into \( E. \) coli lipids (B), followed by incubation on ice for 5 min. ATPase specific activity was determined at 37 °C in the presence of 5 mM ATP. Data points represent the mean ± S.E. (n = 3), and where not visible, error bars are contained within the symbols.

![Graph B](image)

**FIGURE 7.** Fluorescence emission spectra for purified MsbA and NATA. Corrected fluorescence emission spectra are shown for purified MsbA (100 \( \mu \)g/ml) in DM buffer (solid line, \( \lambda_{\text{max}} = 325 \) nm), MsbA following treatment with 6 \( M \) GdnHCl (short dash, \( \lambda_{\text{max}} = 342 \) nm), MsbA following treatment with 6 \( M \) GdnHCl and incubation for 20 min at 100 °C (dotted line, \( \lambda_{\text{max}} = 345 \) nm), and 30 \( \mu \)M NATA (long dash, \( \lambda_{\text{max}} = 348 \) nm). Fluorescence emission was recorded at 20 °C following excitation at 290 nm (2 nm slit widths).

### TABLE 2

| Sample | \( K_{	ext{SV}} \) (M) |
|--------|------------------|
| NATA   | 26.2 ± 1.98      |
| MsbA   | 4.10 ± 0.17      |
| MsbA + ATP | 3.80 ± 0.14    |
| MsbA + lipid A | 3.17 ± 0.13  |
| MsbA + ATP + lipid A | 3.13 ± 0.16  |
| MsbA + H33342 | 1.58 ± 0.03   |
| MsbA + ATP + H33342 | 1.50 ± 0.07 |

Notes:
- NATA (30 \( \mu \)M) or MsbA (100 \( \mu \)g/ml) samples in ATPase buffer were titrated at 10 °C with increasing concentrations of acrylamide solution, in the absence or presence of 2 \( \text{mM} \) ATP and 11 \( \mu \)M lipid A or 10 \( \mu \)M.
- Stern-Volmer quenching constant, determined from the slope of a plot of \( F_0/F \) versus concentration of acrylamide, expressed as values ± fitting error.

H33342 (≈78%) than with nonfluorescent substrates (<50%) (Table 3). This suggests that at least a portion of the quenching for fluorescent compounds is a result of FRET from Trp residues to the bound compound. Nonfluorescent lipid substrates had the lowest maximal quenching (<20%; Table 3). The quenching in this case is likely due solely to conformational changes in the protein upon binding that alter the environment of the fluorescing Trp residues.

Neither colchicine nor daunorubicin modulated the ATPase activity of MsbA (Table 1) but did quench the protein saturably, albeit with the lowest affinity of the compounds examined (≈52 and 31 \( \mu \)M, respectively; see Table 3). Lipid-based drugs such as ilmofosine and D-20133 bound to MsbA with very high affinity, ≈2–3 \( \mu \)M, respectively; Table 3). The potent Pgp modulator LY335979 (Zosuquidar) bound to MsbA with the highest affinity of the compounds tested, despite inhibiting the ATPase activity of the protein significantly only at high concentrations. The Trp fluorescence of MsbA reconstituted into proteoliposomes of \( E. \) coli lipid was saturably quenched by H33342 (Fig. 10D) with a \( K_{	ext{SV}} \) value of 8.8 \( \mu \)M, which is similar to but slightly higher than that measured for H33342 binding to MsbA in detergent solution (Table 3).

### DISCUSSION

MsbA was initially identified as a gene product capable of restoring normal growth to mutants defective in LPS biosynthesis (3). The HtrB protein of \( E. \) coli is a lauroyltransferase involved in the biosynthesis of lipid A (27). Mutations in \( htrB \) cause an overproduction of phospholipids, accumulation of under-acylated derivatives of lipid A, and render the cell unable to grow above 32 °C (28–30). The \( msbA \) gene was identified as one of two multicopy suppressors of \( htrB \) gene insertion mutations (termed \( msbA \) and \( msbB \)) (3, 31). Overexpression of MsbA compensates for the lethal accumulation of lipid A derivatives in the cytoplasmic membrane and results in their appearance in the outer membrane, suggesting that it may be involved in mediating the transport of lipid A and related species to the outer membrane.

Vanadate-sensitive ATPase activity is a hallmark of many ABC proteins. When MsbA was overexpressed in \( E. \) coli, the resulting membrane preparation displayed relatively high ATPase activity that was inhibited 65–70% by \( V_5 \). It is likely that a large fraction of this activity arises from the presence of 5–10% MsbA in the cytoplasmic membrane proteins of the bac-
We successfully isolated MsbA from this membrane preparation to >90% purity and functionally reconstituted the protein into proteoliposomes of *E. coli* lipids, which are suitable for a variety of functional measurements. Homodimerization of MsbA is expected to be a requirement for ATPase activity, because the nucleotide-binding site of ABC proteins is located at the interface between the two NB domains (24). Gel filtration size analysis indicated that MsbA purified using DM displayed a molecular size compatible with that of a dimer, and a faint band with the molecular weight expected for the dimer was also visible on SDS-PAGE.

The ATPase-specific activity of purified MsbA was ~0.9–1.0 μmol/min/mg protein, which is 25–90-fold higher than values reported previously (9, 18) but in agreement with more recent work (5), and suggests that the protein is highly functional. We observed maximal activity at >5 mM ATP, with half-maximal activity at ~2 mM, which is close to the previously reported *K*_m* values for ATP hydrolysis of 0.9 and 1.4 mM (9, 18). Previous work on other ABC proteins has shown that different isolation conditions and detergent concentrations may lead to variations in both *K*_m* and *V*_max* values for ATP hydrolysis. The ATPase activity of our reconstituted MsbA preparation was inhibited by ~80% by *V*_p* with 15 μM *V*_p* causing half-maximal inhibition. This is in good agreement with the results of Doerrler and Raetz (9), who previously reported that 70% of MsbA ATPase activity was vanadate-sensitive in proteoliposomes, with half-maximal inhibition at ~35 μM. As expected from observations made for other ABC transporters, the trapping reagents AlF₃ and BeF₂ also inhibited MsbA ATPase activity.

The ATPase activity of many ABC proteins can be stimulated and/or inhibited by the presence of their substrates. The activity of MsbA in DM solution was stimulated more than 2-fold by the presence of the putative substrate, lipid A, suggesting that this lipid interacts with the protein. Other researchers have shown 2.5–3.5-fold stimulation of MsbA ATPase activity by lipid A (9, 18). Several compounds that are known substrates for
other ABC drug efflux pumps were found to modulate the ATPase activity of MsbA, suggesting that they also interact with the protein. Doerrler and Raetz (9) reported no stimulation by colchicine, daunorubicin, doxorubicin, verapamil, or vinblastine in reconstituted proteoliposomes. In contrast, Reuter et al. (10) reported that the activity of MsbA could be stimulated by daunorubicin, vinblastine, and H33342 in bacterial membrane vesicles. We observed stimulation of MsbA in DM solution for verapamil, inhibition at high concentrations of vinblastine, both stimulation and inhibition by H33342, but no stimulation or inhibition by colchicine and daunorubicin (which are likely to interact with the protein as indicated by Trp quenching).

The intrinsic Trp fluorescence emission spectrum of MsbA was greatly red-shifted when the protein was denatured using GdnHCl and high temperature, approaching the value seen for a soluble Trp analog. The MsbA emission spectrum suggests that the Trp residues contributing to fluorescence are likely to be membrane-embedded and/or located in a nonpolar region within the folded protein. This is compatible with their proposed location within the interfacial region of the membrane near the N terminus.

Dynamic quenchers such as acrylamide report on changes in the solvent exposure of Trp residues, and can provide useful information about conformational changes that take place on binding of substrates. Crystallographic studies of E. coli MsbA suggest a resting state in which the NB domains of the two monomers are separated by ~50 Å in the absence of substrate and nucleotide (5). A large scale movement of protein domains upon nucleotide binding would thus be required to produce catalytically active MsbA, because ATP hydrolysis in ABC proteins occurs at the dimer interface of the two NB domains. Buchaklian et al. (18) used EPR spectroscopy and chemical cross-linking to provide support for this structural model. Here we show that on addition of ATP to MsbA in DM solution, the $K_{SV}$ for quenching by acrylamide showed little change, suggesting that ATP binding does not induce a large scale protein reorientation involving the fluorescent Trp residues. Addition of lipid A to MsbA decreased the $K_{SV}$ value, indicating that the Trp residues become somewhat less accessible to solvent in the presence of the putative physiological substrate. In the presence of both ATP and lipid A, the solvent accessibility was decreased by roughly the same amount as seen for addition of lipid A alone. Our data support the possibility of only a small conformational change upon binding of substrates. ATP binding either in the absence or presence of substrate does not alter Trp solvent accessibility sufficiently to
suggest a large scale re-orientation of the kind required to bring
the NB domains 50 Å closer together. These results do not
exclude the possibility that there is a large change in the protein
conformation (such as a domain rotation) upon substrate
and/or ATP binding that does not alter the solvent exposure of
the Trp residues.

Quenching of intrinsic Trp fluorescence can be a useful tool
for studying ligand binding to proteins. Using this approach, we
report here, for the first time, estimates of the binding affinity of
MsbA for nucleotides, lipids, lipid-like compounds, and a vari-
ey of drugs. ATP, ADP, AMP-PNP, and AMP-PCP all showed
saturable quenching of MsbA Trp fluorescence, and fitting of
the data to an equation yielded $K_d$ values in the range 0.5–2.1
mM. Fluorescent TNP-labeled nucleotides bound to MsbA with
substantially higher affinity, with $K_d$ values of 30–50 µM, simi-
lar to their affinity for binding to Pgp (23). The TNP group likely
enhances binding by providing additional interactions in the
nucleotide-binding site. All the nucleotides appeared to inter-
act with MsbA in the same way as ATP, because they were
either hydrolyzed themselves or competed effectively with ATP
for hydrolysis.

The putative physiological substrate, lipid A, also quenched
MsbA Trp fluorescence with a $K_d$ for binding of 6.4 µM, and
ReLPS, RaLPS, and lipid-based compounds (ilmofosine,
D020133, and D-2166) gave $K_d$ values in the range 2–6 µM.
The relatively high binding affinities found for lipid A and lip-
d-like compounds support the view that MsbA may be a lipid
transporter or flipase. Reuter et al. (10) were the first to show
binding to MsbA of the photoactive Pgp substrate [3H]azido-
planine. This study is the first to estimate the binding affinity of
MsbA for a variety of drugs, with $K_d$ values in the range 0.25–50
µM. Colchicine and daunorubicin bind with lower affinity than
lipids and other MDR drugs, such as H33342 (a reported trans-
port substrate) and LY335979. Several drugs that quench MsbA
Tryp fluorescence (colchicine, daunorubicin, and LY335979) do
not modulate MsbA ATPase activity, which suggests that they
may interact with the protein in a different mode from drugs
that stimulate ATPase activity, such as H33342. If MsbA has
a multisite substrate binding pocket of the type proposed for
Pgp, then some sub-sites may be only very weakly linked to the
NB domains.

The $\Delta F_{\text{max}}$ (maximal fluorescence quenching) values
were very high for TNP-nucleotides, reflecting the fact that quench-
ing of Tryp residues by FRET can take place for these com-
ounds, as documented previously for Pgp (23). The spectral
overlap integral of TNP and Tryp residues is quite high, and for
Pgp there is a strong correlation between spectral overlap and
$\Delta F_{\text{max}}$ (23), which is expected to be true for MsbA as well.
$\Delta F_{\text{max}}$ values were generally low for lipid A and other lipid-
based compounds, but they were substantially higher for sev-
eral drugs, including daunorubicin and H33342. It is possible
that FRET also contributes to Tryp quenching by these drugs.

In general, the $K_d$ values for binding of drugs to MsbA mirror
the values for binding to Pgp (22, 23). For example, the $K_d$ value
for colchicine binding to Pgp is 75 µM. For MsbA, and
0.5 and 1.7 µM for vinblastine binding to Pgp and MsbA, respec-
tively. Although there are no other reports of $K_d$ values for
binding of drugs to MsbA, some transport data exist. Reuter
et al. (10) observed ATP-dependent transport of H33342 in
proteoliposomes, and Woebking et al. (11) further character-
ized the drug transport functions of MsbA. They estimated a
$K_m$ value for H33342 of 0.2–0.52 µM in inside-out membrane
vesicles (11). In comparison, we found an $SC_{\text{max}}$ value of 7.5 µM
for ATPase stimulation of MsbA in proteoliposomes, and a $K_d$
value for binding to MsbA in detergent solution of 6.6 µM.

In this study, we have assumed that substrates interact with
MsbA at an otherwise unoccupied binding site. It is possible
that some endogenous lipid species co-purify with MsbA and
may occupy its binding site. The $K_d$ values determined here
could thus represent either binding to an unoccupied site or
competition with endogenous lipid for binding to an occupied
site.

There appear to be significant similarities between the struc-
ture and function of MsbA and Pgp. The ATPase activity of
both proteins is stimulated by lipid A, RaLPS, and ReLPS, as
shown here and in our previous work (22). Their ATPase activ-
ity can be modulated by MDR drugs, and binding of nucleotides
and drugs quenches the protein Tryp fluorescence. They appear
to be able to act as both lipid flippases and drug transporters.
The dual functional nature of these ABC proteins may have
arisen because of the nonpolar nature and membrane localiza-
tion of their substrates. It seems likely that MsbA handles its
substrates in a similar fashion to Pgp, but much more work will
be required to understand the molecular mechanism of its lipid
A flipase activity.

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