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ATPase Activity of the MsbA Lipid Flippase of *Escherichia coli**

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***Escherichia coli* MsbA, the proposed inner membrane lipid flippase, is an essential ATP-binding cassette transporter protein with homology to mammalian multidrug resistance proteins. Depletion or loss of function of MsbA results in the accumulation of lipopolysaccharide and phospholipids in the inner membrane of *E. coli*. MsbA modified with an N-terminal hexahistidine tag was overexpressed, solubilized with a nonionic detergent, and purified by nickel affinity chromatography to ~95% purity. The ATPase activity of the purified protein was stimulated by phospholipids. When reconstituted into liposomes prepared from *E. coli* phospholipids, MsbA displayed an apparent K_m of 878 μM and a V_{max} of 37 nmol/min/mg for ATP hydrolysis in the presence of 10 mM Mg^{2+}. Preincubation of MsbA-containing liposomes with 3-deoxy-D-mannooctulosonic acid $(Kdo)_2$-lipid A increased the ATPase activity 4–5-fold, with half-maximal stimulation seen at 21 μM Kdo_2-lipid A. Addition of Kdo_2-lipid A increased the V_{max} to 154 nmol/min/mg and decreased the K_m to 379 μM. Stimulation was only seen with hexaacylated lipid A species and not with precursors, such as diacylated lipid X or tetraacylated lipid IV_A. MsbA containing the A270T substitution, which renders cells temperature-sensitive for growth and lipid export, displayed ATPase activity similar to that of the wild type protein at 30 °C but was significantly reduced at 42 °C. These results provide the first *in vitro* evidence that MsbA is a lipid-activated ATPase and that hexaacylated lipid A is an especially potent activator.**

The envelope of *Escherichia coli* and other Gram-negative bacteria consists of an inner membrane, a periplasmic space, and an outer membrane (1). The outer leaflet of the outer membrane is composed of lipopolysaccharide (LPS),[1] a complex glycolipid that is essential for an effective permeability barrier and is a potent stimulator of the innate immune system in mammals (2–5). Lipid A is the hydrophobic anchor of LPS (2–5). Lipid A biosynthesis is essential for survival of most Gram-negative bacteria and is a target for the design of new antibiotics (5–9).

The enzymes that catalyze the synthesis of Kdo_2-lipid A, the minimal lipid A required for survival in laboratory strains of *E. coli*, are cytoplasmic or associated with the inner membrane (2, 3, 5). The mechanism by which *E. coli* transports newly synthesized lipid A from its site of biosynthesis at the inner face of the inner membrane to the outer membrane is not fully understood. Recent studies from our laboratory have demonstrated that export of both lipid A and glycerophospholipids requires the inner membrane protein MsbA (10, 11), originally identified as a multicopy suppressor of the temperature-sensitive growth phenotype of *lpxL*(*htrB*) knockouts (12), a gene encoding a key late acyltransferase in the lipid A biosynthetic pathway (13, 14) (Fig. 1*A*). MsbA is a member of the ATP-binding cassette (ABC) superfamily, is closely related to mammalian multidrug-resistance (MDR) proteins, and is required for growth of *E. coli* (12, 15). Zhou *et al.* (10) demonstrated that depletion of cellular MsbA resulted in the gradual accumulation of lipid A and glycerophospholipids in the inner membrane. More recently, we have isolated a novel, temperature-sensitive *E. coli* point mutant of MsbA (designated WD2) and have shown that loss of MsbA function results in rapid cessation of cell growth, accompanied by accumulation of lipid A and phospholipids in the inner membrane and formation of inner membrane invaginations visible by electron microscopy (11). MsbA in WD2 contains a single amino acid substitution (A270T) located in its fifth transmembrane span (Fig. 1*B*). Our genetic evidence strongly suggests that MsbA functions as a general lipid transporter, or flippase, playing a key role in the movement of lipids from the inner membrane to the outer membrane of *E. coli* (11). Although there is no evidence that MsbA confers multidrug resistance in *E. coli*, the related LmrA protein of *Lactococcus lactis* does in fact confer resistance to many of the same amphiphilic compounds that are transported by MDR1 (16, 17).

E. coli msbA encodes a 584-amino acid residue inner membrane protein (11, 12). It was predicted to contain a classical ABC domain at its C terminus and a hydrophobic region at its N terminus, consisting of six transmembrane spans (11, 12). The proposed topology and dimeric structure of MsbA were validated recently by the crystal structure (18) at 4.5 Å resolution (Fig. 1*B*). MsbA contains an intriguing cleft, oriented toward the cytoplasm, between its two subunits (Fig. 1*B*) (18). The cleft is lined with basic amino acids, and it is sufficient in size to accommodate lipid A or several glycerophospholipid molecules (18). ATP binding and hydrolysis presumably drive a conformational change that results in lipid extrusion to the periplasmic surface of the inner membrane (Fig. 1) (18). The A270T substitution is situated at a critical site near the periplasmic surface in the MsbA molecule (Fig. 1*B*), consistent with the lipid transport defect seen in WD2 at 42 °C.

Previous studies have shown that the intrinsic ATPase activity of MDR proteins and other ABC transporters, when purified and reconstituted into liposomes, can be stimulated by physiologic concentrations of transported substrates (19–23). To develop functional assays for MsbA and to elucidate its role in lipid export, we have expressed the protein as an N-terminal

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[1] The abbreviations used are: LPS, lipopolysaccharide; ABC, ATP-binding cassette; DDM, dodecyl maltoside; His_6, hexahistidine; Kdo, 3-deoxy-D-mannooctulosonic acid; LDAO, *N*, *N*-dimethyldodecylamine *N*-oxide; MDR, multidrug resistance.

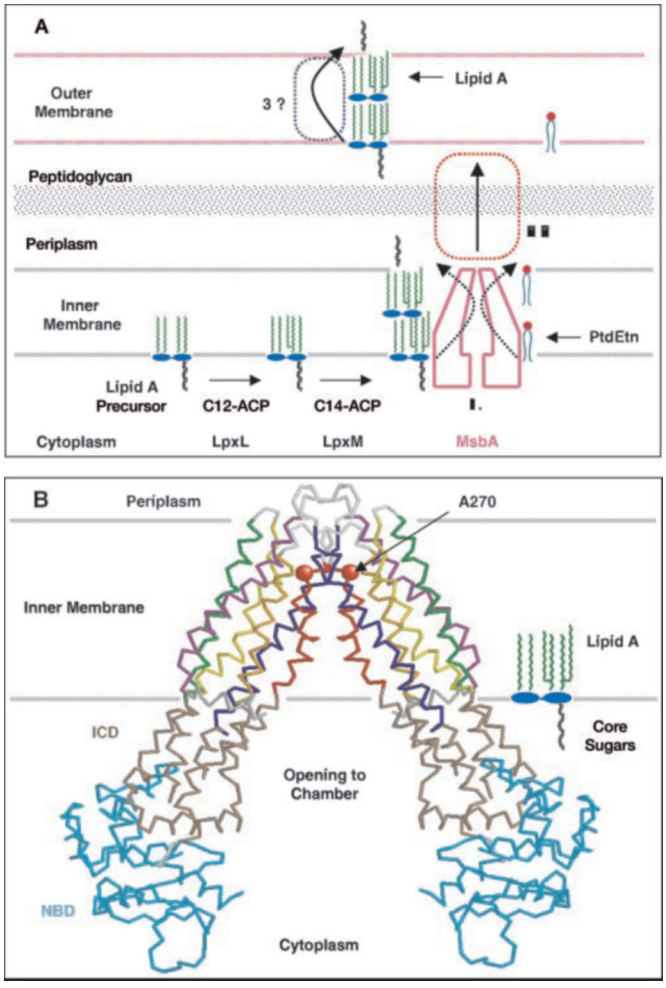

FIG. 1. **Proposed role of *E. coli* MsbA in phospholipid export and structure of the *E. coli* MsbA dimer at 4.5 Å resolution.** *A*, after the MsbA mediated flip-flop/transport of newly made hexaacylated lipid A and phospholipids at the inner membrane (11), additional proteins are likely to be involved in steps 2 and 3, but these have not yet been identified. *B*, this backbone tracing of MsbA was made from Protein Data Bank file 1JSQ (18). Transmembrane helices 1–6 are colored *purple*, *blue*, *yellow*, *green*, *red*, and *orange*, respectively. The intracellular domain (*ICD*) is *brown*, and the nucleotide binding domain (*NBD*) is *cyan* (18). A schematic model of lipid A is shown for size comparison. The location of the Ala-270 residue, which is changed to threonine in the temperature-sensitive lipid transport mutant WD2 (11), is shown as a *red sphere*. The putative chamber for the binding of lipids on the inner surface of the inner membrane (18) is lined with basic residues (not shown).

hexahistidine fusion, purified it to near homogeneity, and reconstituted it into liposomes. The ATPase activity of purified MsbA is dependent upon the presence of phospholipids and is stimulated further by Kdo_2-lipid A.

EXPERIMENTAL PROCEDURES

Materials—LDAO was purchased from Fluka Biochemika. DDM was purchased from Sigma. *E. coli* phospholipids were purchased from Avanti Polar Lipids, Inc. Bio-Beads were from Bio-Rad, and nickel-nitrilotriacetic acid-agarose resin was from Qiagen. Tryptone and yeast extract were from Difco. All other chemicals were reagent grade and purchased from either Sigma or Mallinckrodt.

Plasmid Construction—The *E. coli msbA* gene was amplified from W3110 genomic DNA by PCR with *Pfu* turbo (Stratagene) according to the manufacturer's instructions. The forward primer was 5′-ATG CTG GTT TTT CAT *ATG* CAT AAC GAC-3′ (*Nde*I site underlined), and the reverse primer was 5′-CGG GAT CCT CGA G*TC* ATT GGC CAA ACT GCA TTT TG-3′ (*Bam*HI site underlined). The *msbA* initiation and termination codons in the primers are italicized. These primers were used at a final concentration of 2.5 ng/μl in a 100-μl PCR mixture containing 100 ng of genomic DNA and 5 units of *Pfu* polymerase. The reaction conditions were as follows: a 94 °C denaturation for 1 min followed by 25 cycles of 94 °C (denature) for 1 min, 55 °C (anneal) for 1 min, and 72 °C (extension) for 2 min. This was followed by a 10-min runoff at 72 °C. The gel-purified PCR product was digested with *Nde*I and *Bam*HI (New England Biolabs) and ligated into an *Nde*I/*Bam*HI-digested and shrimp alkaline phosphatase (Roche Molecular Biochemicals)-treated pET28b vector (Novagen) to give vector pWTD1. Vector pWTD7, containing the temperature-sensitive MsbA allele (A270T), was constructed in an identical manner starting from genomic DNA from strain WD2 (11). Vector pWTD3 was constructed by cloning the *Xba*I/*Hin*dIII fragment of pWTD1 (containing the in-frame hexahistidine tag and ribosomal binding site) into an *Xba*I/*Hin*dIII and shrimp alkaline phosphatase-treated vector pACYC184 (New England Biolabs). All plasmids were sequenced at the Duke University sequencing facility and found to be free of errors. Genomic DNA was prepared using the Easy-DNA kit from Invitrogen.

Overexpression of N-His_6-MsbA—pWTD1 was transformed into Novablue (DE3) cells (Novagen). A single kanamycin-resistant colony was inoculated into 5 ml of LB broth (24) (adjusted to pH 7.0 and containing 30 μg/ml kanamycin) and grown at 37 °C overnight. The overnight culture was diluted 100-fold into 500 ml of LB broth containing kanamycin, and the cells were grown at 37 °C until A_{600} reached 0.6. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 1 mM. Growth was continued at 30 °C for 3 h, and cells were harvested by centrifugation at 6,000 × *g* for 15 min. All subsequent steps were carried out at 0–4 °C. Cell pellets were resuspended in 50 ml of 50 mM HEPES buffer, pH 7.5, containing 5 mM β-mercaptoethanol and were broken by passage through a French pressure cell at 18,000 p.s.i. The crude lysate was cleared by centrifugation at 10,000 × *g* for 20 min. Membranes were prepared by two sequential ultracentrifugation steps at 100,000 × *g* for 60 min with a wash of the first membrane pellet in 10 ml of 50 mM HEPES buffer, pH 7.5, 500 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, and 5 mM β-mercaptoethanol (buffer A). The final membrane pellet was homogenized in buffer A at a protein concentration of ~5 mg/ml.

Solubilization and Purification of N-His_6-MsbA—LDAO was added to the washed membranes (5 mg/ml in buffer A) to a final concentration of 2% (w/v) and incubated with rotation for 1 h at 4 °C. Insoluble material was removed by ultracentrifugation at 125,000 × *g* for 1 h. The soluble fraction was adjusted to 10 mM imidazole and loaded onto a preequilibrated nickel-nitrilotriacetic acid-agarose column. After washes with 5 column volumes of 10 mM and then with 5 column volumes of 50 mM imidazole in buffer A containing 0.1% DDM, protein was eluted with 5 column volumes of 200 mM imidazole in buffer A containing 0.1% DDM.

Reconstitution of Purified N-His_6-MsbA—For reconstitution of the purified protein, *E. coli* phospholipids (polar lipid extract, Avanti) were dried under a stream of nitrogen and then subjected to sonic irradiation for 5 min in a bath sonicator at a concentration of 25 mg/ml in 50 mM HEPES, pH 7.5, containing 50 mM NaCl, 5 mM $MgCl_2$, and 2 mM β-mercaptoethanol. The liposomes were frozen on dry ice, thawed slowly at room temperature, and then homogenized by five passages through a 27-gauge needle. The liposomes were diluted to a concentration of 4 mg/ml in the above buffer, and DDM was added to solubilize the system to generate mixed micelles (1 μmol of DDM/mg of lipid). Solubilization was followed spectrophotometrically at 540 nm (25). Purified *N*-His_6-MsbA was added to the lipid/DDM mixed micelles at a protein:lipid ratio of 1:100 (w/w). The mixture was incubated with gentle agitation at room temperature for 30 min. Bio-Beads (previously washed extensively with methanol and then water) were added at 80 mg/ml. The system was incubated for 2 h at 4 °C and then for 2 h and for 16 h at 4 °C after the addition of fresh Bio-Beads. The final proteoliposomes were isolated by ultracentrifugation at 125,000 × *g* for 1 h at 4 °C. They were resuspended at 0.25 mg of protein/ml in buffer A containing 1 mM dithiothreitol instead of β-mercaptoethanol and stored at −80 °C. All ATPase assays were carried out using proteoliposomes that had undergone a freeze/thaw step and had been passed through a 27-gauge needle several times.

ATPase Assay—Purified MsbA protein in 0.1% DDM or reconstituted into vesicles was assayed for ATPase activity at a protein concentration of ~1–10 μg/ml in a 150-μl reaction mixture containing (final concentrations) 50 mM HEPES, adjusted to pH 7.5 with NaOH, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 2 mM ATP. In assays containing 0.1% DDM, 10% glycerol was also included. If exogenous lipids or inhibitors were included in the assay, a 15-min preincubation of all components on ice was included before the incubation at 37 °C. ATPase reactions were incubated for the indicated times at 37 °C and stopped by the addition

TABLE I
Relevant strains and plasmids

Strain or plasmid	Description	Source or reference
E. coli		
W3110	Wild type, F^-, λ^-	*E. coli* Genetic Stock Center, Yale University
WD2	W3110, *aroA::Tn10 msbA2* (A270T)	11
XL1-Blue MR	Δ*mcrABC recA1 endA1 gyrA96* relA1 *supE44 thi-1 lac*	Stratagene
NovaBlue (DE3)	Δ*(srl-recA)306::Tn10*(DE3) Tet^r	Novagen
Plasmid		
pET28b	Expression vector; T7*lac* promoter, Kan^r	Novagen
pACYC184	Low copy vector. Tet^r Cam^r	New England Biolabs
pWTD1	pET28b containing wild type *E. coli msbA* (*Nde*I/*BamH*I sites)	This work
pWTD3	*Xba*I/*Hind*III fragment of pWTD1 cloned into pACYC184	This work
pWTD7	pET28b containing *msbA2* of WD2 (*Nde*I/*BamH*I sites)	This work

of 150 μl of 12% SDS. The amounts of P_i released were determined by a colorimetric method, as described by Gonzalez-Romo *et al*. (26), using potassium phosphate as the standard. After the addition of SDS, 300 μl of a solution containing equal volumes of 12% ascorbic acid in 1 M HCl and 2% ammonium molybdate in 1 M HCl was added, and the samples were incubated at room temperature for 5 min. This was followed by the addition of 450 μl of an aqueous solution of 2% sodium citrate, 2% sodium metaarsenite, and 2% acetic acid. After a 20-min incubation at room temperature, absorbance was measured at 850 nm. The assay was linear in the range of 6–400 μM P_i.

Isolation of Kdo_2-Lipid A—WBB06 cells (27) were grown overnight at 37 °C to A_{600} ~3.5 in 2 liters of LB broth adjusted to pH 7.0 and supplemented with 12 μg/ml tetracycline. Cells were harvested by centrifugation at 6,000 × *g* for 10 min, washed with an equal volume of phosphate-buffered saline (28), and resuspended in 160 ml of phosphate-buffered saline. Chloroform and methanol were added to give a single-phase Bligh-Dyer mixture consisting of chloroform:methanol:phosphate-buffered saline (1:2:0.8, v/v) (29). The cells were extracted for 1 h at room temperature with frequent mixing. Insoluble material was removed by centrifugation at 3,000 × *g* for 10 min, and the supernatant, containing the glycerophospholipids and Kdo_2-lipid A, was removed. It was converted to a two-phase Bligh-Dyer system by the addition of chloroform and water to give the mixture chloroform:methanol:water (2:2:1.8, v/v) (29). After thorough mixing, the phases were separated by centrifugation as described above, and the upper phase was washed with a fresh, preequilibrated Bligh-Dyer lower phase. The lower phases were pooled, washed once with a fresh, preequilibrated Bligh-Dyer upper phase, and dried in a rotary evaporator. The dried lipids were redissolved in 10 ml of chloroform:methanol:water (2:3:1, v/v) and applied to a 2-ml DEAE-cellulose column (Whatman DE52), equilibrated as the acetate form in the same solvent mixture (30, 31). The column was washed with 2 column volumes of chloroform:methanol:water (2:3:1, v/v) and eluted with 5 column volumes each of chloroform:methanol:aqueous ammonium acetate (at 60, 120, 240, 360, or 480 mM) (2:3:1, v/v) (31) while collecting 2-ml fractions throughout the elution. Phosphatidylethanolamine was not bound to the column, whereas phosphatidylglycerol and other phospholipids eluted in the 60–120 mM fractions. The Kdo_2-lipid A 1,4′-*bis*-phosphate species eluted in the 240 mM fraction, but the Kdo_2-lipid A 1-pyrophosphate variant (32, 33) eluted in the 360 mM fractions. The fractions containing the purified Kdo_2-lipid A 1,4′-*bis*-phosphate were pooled and converted to a neutral two-phase Bligh-Dyer system by the addition of appropriate amounts of chloroform and water. The lower phase was neutralized with a few drops of pyridine and was dried under a stream of nitrogen. It was then resuspended with sonic irradiation for 2 min in a bath apparatus in 20 mM HEPES, pH 7.5, and stored at −80 °C. The elution of the various lipids from the DEAE column was monitored by spotting ~5-μl samples onto Silica Gel 60 TLC plates followed by development in a solvent containing chloroform, pyridine, 88% formic acid, and water (50:50:16:5, v/v), and visualization by sulfuric acid charring. The amount of purified lipid was quantified by complete acid hydrolysis in 50 μl of 5 M HCl at 100 °C for 16 h followed by measurement of the released P_i, using the phosphate assay described above (26). Potassium phosphate samples were run in parallel to generate a standard curve. The final yield of Kdo_2-lipid A 1,4′-*bis*-phosphate was ~5 mg from 2 liters of cells. The Kdo_2-lipid A from strain WBB06 was greater than 95% pure as judged by TLC analysis followed by sulfuric acid charring, and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (data not shown).

Lipid X was isolated from *E. coli* MN7 (34) as described by Radika and Raetz (35). Lipid IV_A was prepared according to the procedure of Garrett *et al.* (36) and was a gift from Dr. Stephen Trent of this laboratory. RaLPS (3) was purchased from Sigma and suspended at a concentration of 1 mM (molecular weight ~4,000) in 20 mM HEPES, pH 7.5. Free hexaacylated lipid A was prepared by pH 4.5 hydrolysis at 100 °C from either RaLPS or Kdo_2-lipid A (10), and quantified by phosphate assay as described above. All lipids suspended in aqueous buffers were dispersed before use by sonic irradiation for 1 min in a bath sonicator.

Analytical Methods—Protein samples were denatured at 100 °C for 5 min and analyzed by 12% SDS-PAGE followed by Coomassie staining. Prestained low range standards from Bio-Rad were used to estimate protein molecular weights. Protein concentrations were determined with the Coomassie Plus Protein Assay Reagent (Pierce) using bovine serum albumin as the standard.

RESULTS

Overexpression and Purification of E. coli MsbA—To facilitate purification, the *E. coli msbA* gene was cloned into pET28b behind the T7 promoter in-frame with an N-terminal His_6 tag. This expression plasmid was designated pWTD1 (Table I). The His-tagged protein, when expressed on the low copy vector pWTD3 (a pACYC184 derivative), was functional as judged by its ability to complement the growth at 44 °C of the MsbA temperature-sensitive mutant WD2 (data not shown).

Membranes prepared from cells overexpressing *msbA* on pWTD1 contained large amounts of a protein migrating at a molecular mass of ~65 kDa which was absent in cells harboring pET28b (Fig. 2). The His-tagged MsbA fusion protein was solubilized efficiently from membranes using 2% LDAO and was purified by nickel affinity chromatography (Fig. 2). During chromatography the detergent was changed to DDM. The protein was greater than 95% pure as judged by SDS-PAGE and Coomassie Blue staining (Fig. 2). A typical preparation yielded ~3 mg of purified MsbA protein from 30 mg of washed membranes.

ATPase Activity of Detergent-solubilized, Purified MsbA in the Presence or Absence of Added Lipids—Using a colorimetric assay for released P_i (26), we found that the ATPase specific activity of the purified, detergent-solubilized MsbA was low, typically in the range of 2–4 nmol/min/mg (Fig. 3, *top panel*). The addition of *E. coli* phospholipids to the assay system at concentrations above 2 mg/ml greatly stimulated and stabilized the ATPase activity (Fig. 3, *middle panel*). Phospholipids at 2 mg/ml or above would be expected to form vesicles, even in the presence of 0.1% (1 mg/ml) DDM. However, the addition of Kdo_2-lipid A at low micromolar concentrations also resulted in stimulation of ATPase activity (Fig. 3, *bottom panel*). At this relatively low concentration the lipid should form mixed micelles with the nonionic detergent (37).

ATPase Activity Associated with Purified, Liposome-reconstituted MsbA—In light of the results shown in Fig. 3, we routinely reconstituted the solubilized MsbA protein into liposomes at a protein:lipid ratio of 1:100 (w/w). Proteoliposomes

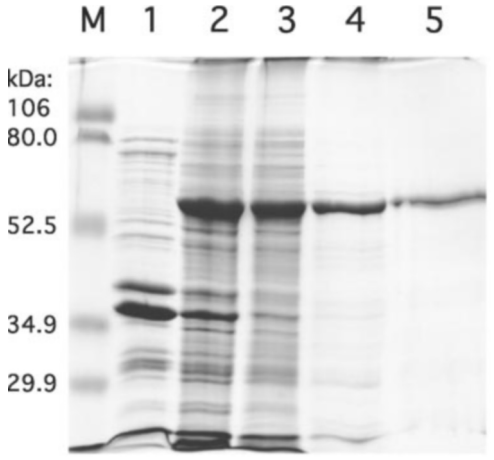

FIG. 2. **Overexpression and purification of *E. coli* MsbA.** The sizes of the molecular mass standards (*lane M*) are indicated to the *left* of the gel. *Lane 1*, membranes (10 μg) from Novablue (DE3)/pET28 vector control cells; *lane 2*, membranes (10 μg) from Novablue (DE3)/pWTD1 cells; *lane 3*, 100,000 $\times$ *g* supernatant after solubilization of membranes with 2% LDAO (applied in a volume equal to that in *lane 2*); *lane 4*, MsbA protein eluted from the nickel column (5 μg); *lane 5*, reconstituted purified MsbA protein (5 μg). After electrophoresis, the gel was stained with 0.25% Coomassie Blue.

were prepared from *E. coli* phospholipids and purified MsbA, both solubilized with DDM, followed by treatment with Bio-Beads to remove the detergent as described under "Experimental Procedures." The MsbA-containing proteoliposomes were found to have an intrinsic ATPase activity that was nearly linear with time at 37 °C for 2 h, and with a protein concentration up to at least 2 μg/150 μl of reaction mixture (Fig. 4, *A* and *B*). Using 2 mM ATP, a magnesium ion concentration of 10 mM (Fig. 4*C*) and pH values ranging from 7 to 8.5 (data not shown) were optimal. The V_{max} of the reconstituted protein was 37 nmol/min/mg, and the apparent K_m for ATP was 878 μM (Fig. 5). These parameters are in the same range as reported for the ABC transporters cystic fibrosis transmembrane conductance regulator (CFTR), multidrug resistance protein 1 (MRP1), and rod photoreceptor-specific ABC transporter (ABCR) (21, 22, 38, 39). Proteoliposomes prepared from mock-purified extracts of vector (pET28b)-containing control cells were found to have a background activity that was indistinguishable from liposomes prepared in the absence of protein (data not shown). Therefore, in the experiments shown in Figs. 4 and 5, reaction mixtures containing protein-free liposomes were run in parallel with the MsbA-reconstituted liposomes and subtracted as nonenzymatic ATPase blanks. In general these no-protein controls represented less than 10% of the activity seen with reconstituted MsbA (data not shown).

Additional Stimulation of MsbA ATPase Activity by Hexaacylated Lipid A—In view of the genetic evidence that MsbA might function both as a phospholipid and as a lipid A transporter (10, 11), we tested the effect of hexaacylated Kdo_2-lipid A on the ATPase activity of reconstituted MsbA in proteoliposomes. Kdo_2-lipid A isolated from a heptose-deficient mutant of *E. coli* (27) was incubated at various concentrations either with protein-free control liposomes or with MsbA-containing proteoliposomes for 15 min at 0 °C immediately before the assay at 37 °C. The ATPase activity of MsbA increased as much as 5-fold over the basal level seen in the absence of Kdo_2-lipid A (Fig. 6*A*). Stimulation was half-maximal at ~21 μM Kdo_2-lipid A (Fig. 6*A*). The data suggest that MsbA may interact with hexaacylated lipid A more strongly than with *E. coli* glycerophospholipids, resulting in additional stimulation of ATPase activity.

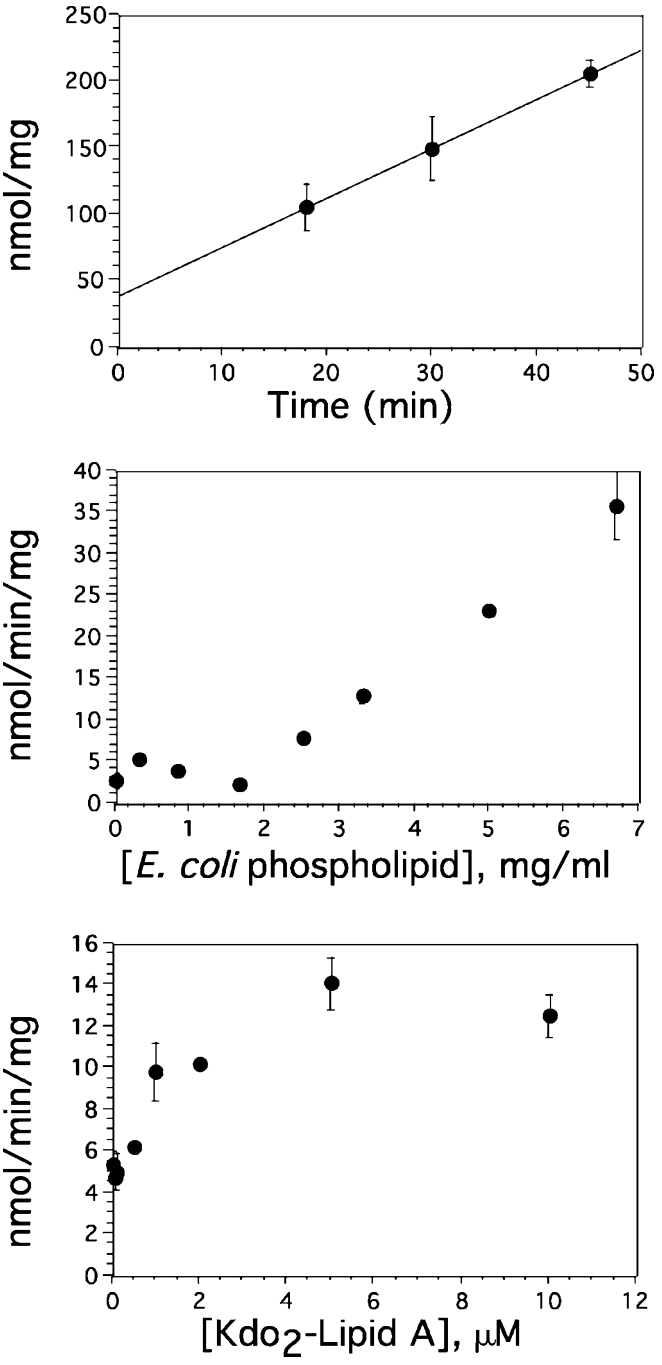

FIG. 3. **ATPase activity of solubilized purified MsbA and its stimulation by *E. coli* phospholipids or Kdo_2-lipid A.** *Top panel*, purified protein (25 μg/ml) was incubated in the presence of 2 mM ATP, 50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% DDM, and 10% glycerol for various times at 37 °C, and the generation of P_i was measured (26). *Middle panel*, *E. coli* phospholipids (Avanti) were dried down under nitrogen and sonicated in aqueous buffer at a concentration of 25 mg/ml. Appropriate volumes were then added to the ATPase assay system described above to achieve the indicated final concentrations of lipid. Incubations were carried out in the presence or absence of added protein (25 μg/ml) for 45 min. The generation of P_i was measured (26), and the background from the protein-free controls was subtracted for each point. *Bottom panel*, Kdo_2-lipid A was added to the ATPase assay system described above in the presence or absence of MsbA protein (25 μg/ml), and the incubation was carried out at 37 °C for 45 min. All values are the averages of triplicate determinations shown with standard deviations.

To investigate the structural specificity of the observed stimulation, MsbA proteoliposomes were preincubated with other LPS preparations or with lipid A precursors, each at ~100 μM,

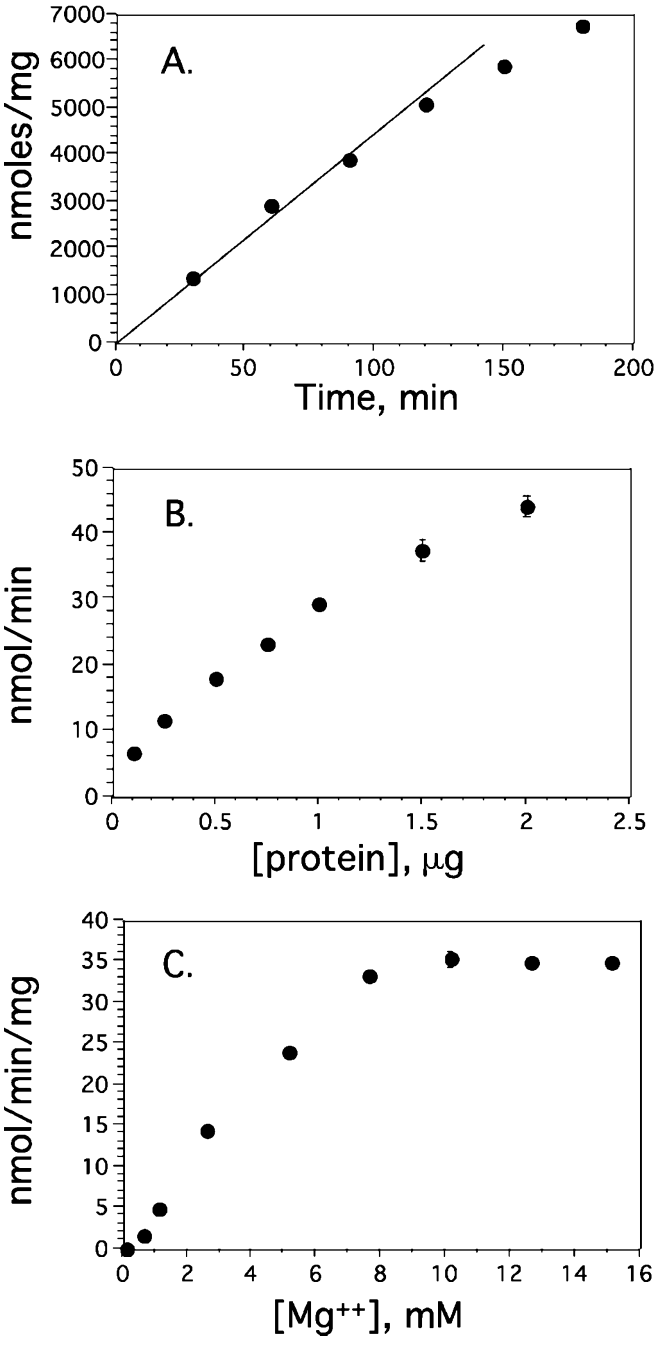

FIG. 4. **ATPase activity of MsbA reconstituted into proteoliposomes.** *A*, proteoliposomes (containing 1 μg of purified MsbA protein) were incubated in a 150-μl final volume consisting of 2 mM ATP, 50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, and 1 mM dithiothreitol for various times at 37 °C, and generation of P_i was measured (26). *B*, increasing amounts of protein were incubated for 2 h at 37 °C in the above ATPase assay mixture, and P_i generation was measured. *C*, proteoliposomes (1 μg of purified MsbA protein/150 μl) were incubated in the standard ATPase assay mixture with different amounts of $MgCl_2$ at 37 °C, and P_i generation was measured. Control liposomes lacking protein were incubated in parallel, and nonenzymatic P_i release was subtracted from the values obtained in the presence of the MsbA-containing proteoliposomes. All values are the averages of triplicate determinations shown with standard deviations.

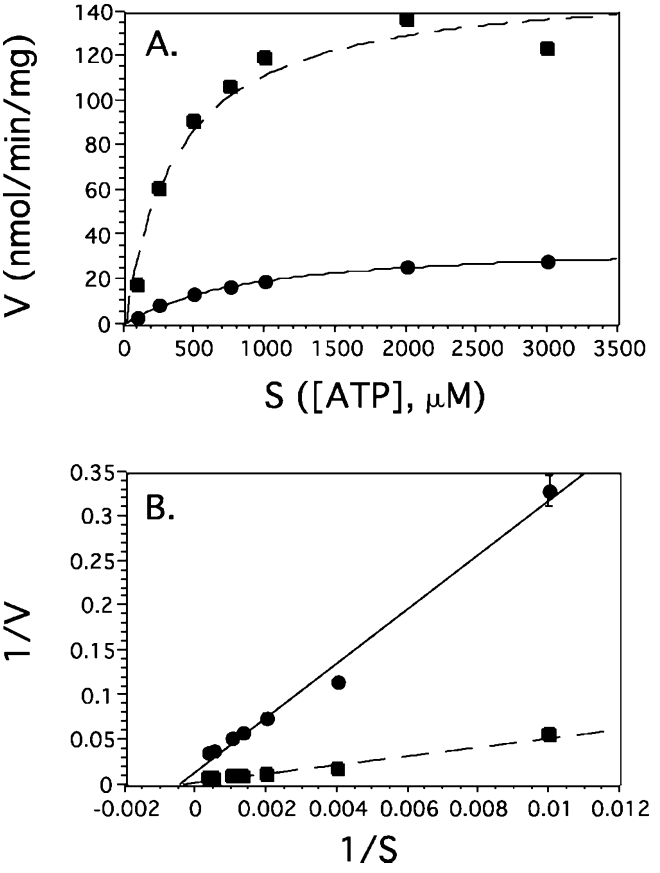

FIG. 5. **Apparent K_m and V_{max} of purified MsbA for ATP and the effect of 50 μM Kdo_2-lipid A.** Proteoliposomes (1 μg/150 μl of reaction mixture) were preincubated on ice for 15 min with 50 μM Kdo_2-lipid A (*squares*) or without Kdo_2-lipid A (*circles*) in standard assay buffer containing the indicated concentrations of ATP (100–3,000 μM) immediately before incubation at 37 °C for 2 h. All values are the averages of triplicate determinations shown with standard deviations. In *A*, apparent K_m and V_{max} values were determined using nonlinear least squares fitting to the equation $V = (V_{max}[S])/(K_m + [S])$. Similar K_m and V_{max} values were obtained from the Lineweaver-Burk plot (*B*).

as well as with phosphatidylcholine and cholesterol, which are not made by *E. coli*. As illustrated in Fig. 6*B*, the precursors lipid X and lipid IV_A (Fig. 7) do not stimulate the ATPase activity of MsbA above the levels achieved with glycerophospholipids alone. However, purified hexaacylated lipid A (lacking the Kdo region and other core sugars), obtained by pH 4.5 sodium acetate hydrolysis of LPS, also stimulates the ATPase activity of MsbA above the level seen with reconstituted liposomes (Fig. 6*B*). Commercially available LPS from *E. coli* EH100 (which contains hexaacylated lipid A and a complete core domain but lacks *O*-antigen) likewise stimulates the ATPase activity of MsbA (Fig. 6*B*) above the level seen with glycerophospholipids. These observations are consistent with previous reports that lipid A precursors are not transported to the outer membrane as rapidly as hexaacylated lipid A is (40, 41).

The V_{max} for ATP calculated in the presence of 50 μM Kdo_2-lipid A increased from 37 to 154 nmol/min/mg, whereas the K_m was decreased by more than half (Fig. 5). Effects of similar magnitude were reported for the V_{max} and K_m of reconstituted ABCR after preincubation with all-*trans*-retinal (21, 22). Taken together with previous genetic studies (10, 11), these results suggest that LPS species containing hexaacylated lipid A are recognized the most effectively by MsbA, although glycerophospholipids are stimulatory as well. The MsbA assays described in Figs. 3–7 should facilitate the search for specific small molecule inhibitors of MsbA, which might be useful as leads for novel antibiotics.

The eukaryotic lipids cholesterol and phosphatidylcholine have no effect in stimulating the MsbA ATPase above the liposome background (Fig. 6*B*). In some mammalian tissues, cholesterol efflux is mediated by the ABCA1 protein (42–44),

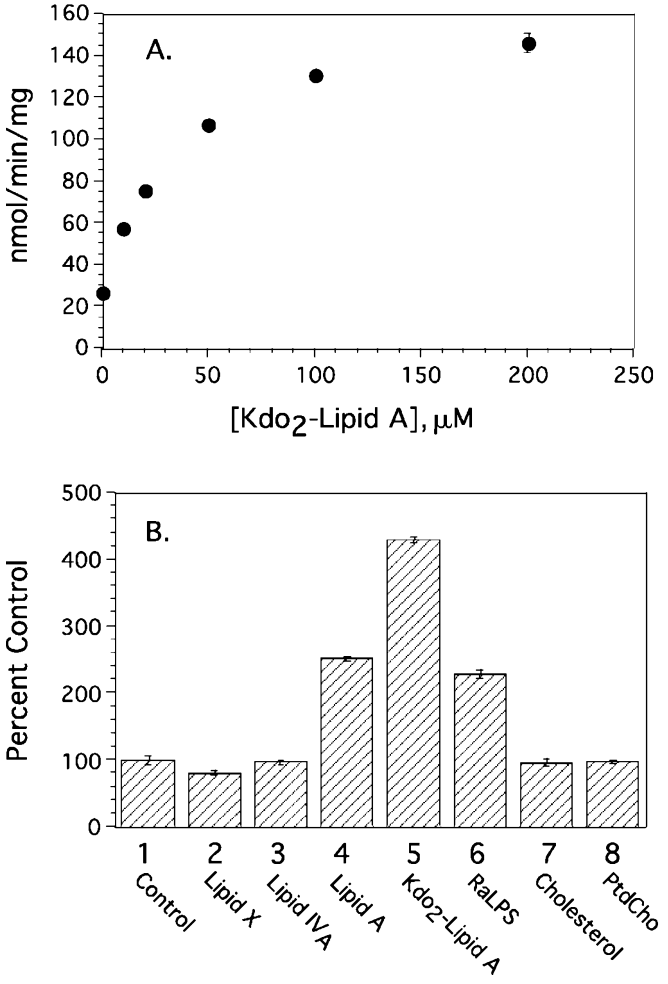

FIG. 6. **Stimulation of MsbA ATPase activity in proteoliposomes by Kdo_2-lipid A and related substances.** *A*, proteoliposomes (1 μg/150 μl of reaction mixture) or protein-free liposome controls were preincubated on ice for 15 min with increasing concentrations of purified Kdo_2-lipid A before a 2-h incubation at 37 °C in the standard ATPase assay buffer. P_i release was measured (26), and values obtained with appropriate blank liposomes were subtracted as background. *B*, proteoliposomes (1 μg/150 μl of reaction mixture) or an equal volume of protein-free liposomes was preincubated on ice for 15 min with 100 μM lipid A, various lipid A precursors, or other control lipids. P_i release was assayed, and all values are the averages of triplicate determinations shown with standard deviations. Stock solutions of lipid A precursors were dispersed in 20 mM HEPES, pH 7.0, with sonic irradiation before addition to the ATPase assay buffer. The lipids phosphatidylcholine and cholesterol were dissolved in ethanol stock solutions at a concentration of 10 mM and then diluted 1:100 into the ATPase assay buffer. Ethanol controls were run in parallel and found not to inhibit or stimulate MsbA ATPase activity.

an ABC transporter defective in Tangier disease (45–47).

Additional Characterization of the MsbA ATPase Activity—The effects on MsbA of known inhibitors or activators of previously characterized ABC transporters were assessed. Sodium *ortho*-vanadate (or one of its oligomeric forms present in solution) is a potent inhibitor of the P-glycoprotein ATPase. Vanadate is thought to block the catalytic cycle by forming a noncovalent complex with MgADP at the ATPase site of the transporter (48, 49). Vanadate inhibits the ATPase activity of reconstituted MsbA by about 70%, with half-maximal inhibition observed at ~35 μM (Fig. 8). In contrast, sodium azide, an inhibitor of SecA and F-type ATPases (50), had no effect on MsbA (data not shown). Substrates of Mdr1, like verapamil, doxorubicin, vinblastine, daunomycin, or colchicines (16, 51), likewise had no effect on the ATPase activity of MsbA (data not shown).

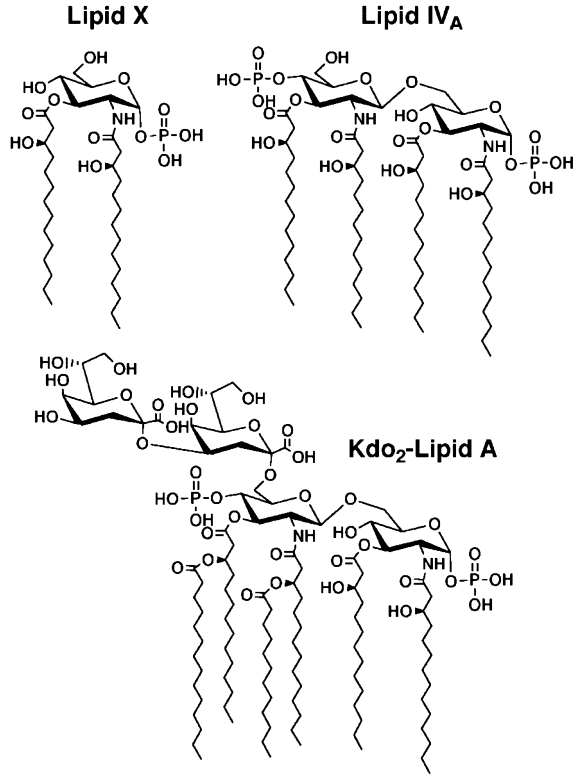

FIG. 7. **Structures of lipid X, lipid IV_A, and Kdo_2-lipid A.** The roles of these substances as LPS precursors are well established (3, 5).

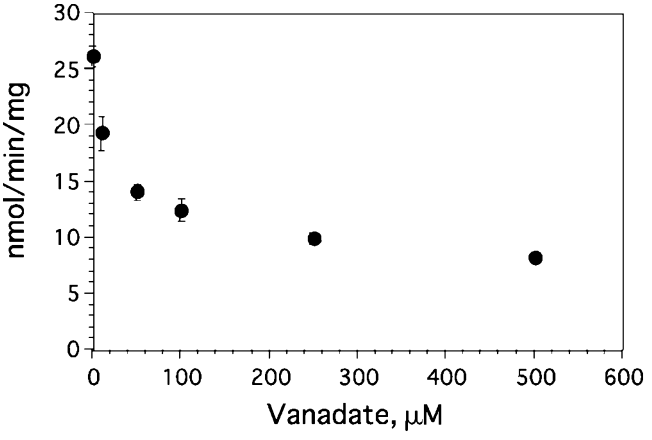

FIG. 8. **Inhibitory effect of vanadate on the ATPase activity of MsbA.** Proteoliposomes (1 μg/150 μl of reaction mixture) were preincubated on ice for 15 min with increasing amounts of sodium *ortho*-vanadate (10–500 μM) in the standard ATPase assay buffer before incubation at 37 °C for 2 h. All P_i release values are the averages of triplicate determinations shown with standard deviations.

Effect of the A270T Mutation on the ATPase Activity of MsbA—The mutant WD2 contains a single amino acid substitution in MsbA (A270T) (Fig. 1*B*), which renders the cells temperature-sensitive for growth (11). WD2 cells stop dividing 1 h after a shift from the permissive temperature of 30 °C to 42 or 44 °C (11). Growth inhibition is accompanied by the accumulation of newly made lipid A and glycerophospholipids in the inner membrane (11). Concurrently, multiple inner membrane folds and invaginations become visible by electron microscopy (11).

MsbA (A270T) was amplified from WD2 genomic DNA, overexpressed, purified, and reconstituted in the same manner as wild type MsbA. The basal ATPase specific activities of both the wild type and mutant proteins were measured at 30 and 42 °C.

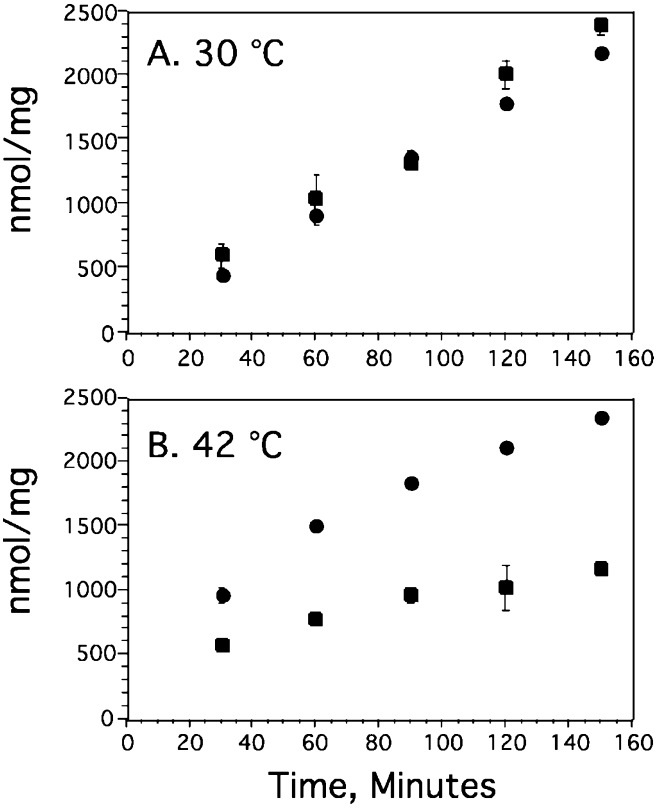

FIG. 9. **Reduced ATPase activity of reconstituted mutant A270T MsbA at 42 °C.** Proteoliposomes (1 μg of purified MsbA protein/150 μl of reaction mixture), containing either wild type (*circles*) or mutant A270T MsbA (*squares*), were incubated for increasing times in the standard ATPase assay buffer at 30 °C (*A*) or at 42 °C (*B*). All P_i release values are the averages of triplicate determinations shown with standard deviations.

Interestingly, although both proteins had similar ATPase activities at 30 °C, the mutant activity was decreased significantly at 42 °C (Fig. 9). However, the -fold stimulation by Kdo_2-lipid A was similar for both proteins at both temperatures (data not shown).

DISCUSSION

Previous studies from our laboratory have shown that depletion of cellular MsbA or the presence of a conditionally inactivating mutation in MsbA results in the loss of transport of lipid A and glycerophospholipids from the cytoplasmic membrane to the outer membrane (10, 11). These observations suggest that MsbA functions either as a general lipid flippase, catalyzing the transbilayer movement of lipids, or is involved in initiating the transperiplasmic movement of LPS and phospholipids, or both.

Our findings in *E. coli* support the idea of a more general function for eukaryotic and other MDR-like proteins, to which MsbA is closely related, in the transport of membrane lipids. However, because the three major MDR proteins of animal cells are not essential for cell growth (51) and because mutations do not result in a generalized lipid transport defect, genetic approaches have not been very revealing so far. One important exception is the Mdr2-deficient mouse, which is characterized by a complete absence of phosphatidylcholine in its bile, resulting in liver disease (52), suggesting that MDR2 is a phosphatidylcholine-specific transporter in cells lining the biliary tree. Another relevant example is the selective lipid export function of the ABCA1 protein, which is defective in Tangier disease (45–47). Fibroblasts from patients with Tangier disease appear to have a defect in the export of cellular cholesterol and phospholipids from the plasma membrane to circulating high density lipoproteins (53, 54).

The presence of dozens of additional uncharacterized membrane proteins that are related to the conventional MDRs in animal cells may explain the lack of generalized lipid transport phenotypes seen in the available MDR knockout mice. Some of these additional MDR-like proteins may be able to catalyze lipid transport reactions similar to those catalyzed by the major MDR proteins (55), and they might function singly or in combination.

Several ABC transporters of the MDR family have already been shown to possess intrinsic ATPase activity that is stimulated in the presence of transported substrates. The best example is the mammalian Mdr1 (P-glycoprotein), which possesses an ATPase activity that is stimulated 3–4-fold by various drugs that it is known to transport, including doxorubicin and vinblastine (17, 19). Similarly, the ABCR protein has an ATPase activity that is stimulated 3–4-fold by all-*trans*-retinal and *N*-retinylidene-phosphatidylethanolamine (21, 22). ATPase activity has also been reported for the transporter associated with antigen processing (TAP), which is stimulated by peptides (23).

In an effort to study the biochemistry of *E. coli* MsbA, we have now purified and reconstituted the protein and have characterized its ATPase activity for the first time. The pure protein, when reconstituted with *E. coli* phospholipids, possesses apparent K_m and V_{max} values for ATP which are similar to those reported for several other purified ABC transporters (21, 22, 38, 39). Interestingly, micromolar concentrations of hexaacylated lipid A, Kdo_2-lipid A, and LPS with an intact core stimulate the basal ATPase activity above the level achieved by glycerophospholipids alone. This stimulation appears to be specific because it is not seen with the same concentrations of lipid X or lipid IV_A. The latter are lipid A precursors (Fig. 7) that contain either two or four acyl chains (5, 31, 56, 57), and they are transported to the outer membrane less efficiently than is hexaacylated lipid A (40, 41).

Our prior studies have shown that all phospholipids and lipid A are retained in the inner membrane after the loss of function of MsbA (10, 11). In the present work, detergent-solubilized MsbA exhibited a low basal ATPase activity that was greatly stimulated by the addition of phospholipids (Fig. 3). When MsbA was reconstituted into proteoliposomes of *E. coli* phospholipids in the absence of lipid A, significant ATPase activity was likewise observed (Fig. 4). However, when lipid A was added to these proteoliposomes, a large increase in ATPase activity was seen (Figs. 5 and 6). These data are consistent with the hypothesis that MsbA is both a phospholipid and a lipid A pump which couples ATP hydrolysis to lipid efflux. MsbA may bind to lipid A more effectively than to glycerophospholipids because of the additional intermolecular contacts that could be achieved with a hexaacylated *versus* a diacylated lipid. In wild type cells, the entire core domain would be attached to lipid A prior to flip-flop (2, 3), and its presence would have to be accommodated by MsbA.

Recent experiments designed to study phospholipid flip-flop in bacterial membranes and model liposomes have suggested that this process might be energy-independent in bacteria (58, 59). In model membranes, lipid flip-flop occurs at near physiological rates in the absence of ATP or a proton motive force, provided that synthetic membrane-spanning peptides are reconstituted together with the lipids (60). These findings would appear to be at odds with the our results from mutant WD2, in which lipid transport is arrested at the nonpermissive temperature despite the presence of a normal complement of inner membrane proteins (11). One explanation might be that MsbA

uses energy from ATP hydrolysis to pump lipids from the outer face of the inner membrane to the outer membrane while facilitating generalized lipid flip-flop in an energy-independent manner (Fig. 1*A*). Alternatively, extrapolations from studies with isolated or reconstituted membranes may not adequately recapitulate the intracellular environment.

The recent x-ray structure of MsbA (18) demonstrates that the protein forms a homodimer (Fig. 1*B*). The dimer contains a cleft, facing the cytoplasm in the available structure (18), through which newly synthesized lipids might pass before export (Fig. 1*B*). A conformational change caused by lipid binding to the membrane-embedded portion of the cleft (Fig. 1*B*) might be coupled to ATP hydrolysis and transport or *vice versa*. This model is consistent with our biochemical data (Figs. 3–6) as well as with studies of other MDR proteins. The A270T mutation in WD2 appears to lie near the periplasmic interface of the two monomers (Fig. 1*B*). One might speculate that MsbA (A270T) cannot form functional dimers at the nonpermissive temperature or that the dimer interface cannot open to expel newly made lipids on the periplasmic surface of the inner membrane.

The availability of purified, catalytically active MsbA lays the groundwork for additional functional studies. If MsbA is indeed a general lipid flippase, as the crystal structure suggests (Fig. 1*B*) (18), this activity might be detectable in proteoliposomes. LPS or phospholipids could be labeled with fluorescent reporter groups and incorporated into proteoliposomes. MsbA could be then tested for its ability to stimulate the loss of quenchable fluorescence by impermeant reagents (60), indicative of flip-flop. This kind of approach was utilized to demonstrate lipid flippase activity of the ABC transporter LmrA of *L. lactis* (61). However, LmrA is not required for growth in its native setting (62),[2] perhaps because of the presence of additional LmrA-like proteins in *Lactococcus* (63).

We have been unable to make *E. coli* cells resistant to several antibiotics, including streptomycin, novobiocin, or erythromycin, following overexpression of MsbA from either low or high copy vectors (data not shown). These observations, coupled with the fact that *msbA* mutations are not known to be associated with generalized antibiotic resistance phenotypes in *E. coli* (17), support the notion that *E. coli* MsbA functions primarily to transport lipid A and glycerophospholipids.

If MsbA is part of a larger molecular machine catalyzing the transperiplasmic movement of phospholipids and LPS, its transport activity might be detectable only in the presence of acceptor vesicles and selected outer membrane and periplasmic proteins. A biochemical assay of this kind might actually lead to the identification and purification of the relevant additional protein components of the transport machinery (Fig. 1*A*). This general strategy has been very successful in the characterization of the LolA-LolE system, which is responsible for transporting bacterial lipoproteins across the periplasm of Gram-negative bacteria (64).

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REFERENCES

1. Nikaido, H. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., ed) 2nd Ed., Vol. 1, pp. 29–47, American Society for Microbiology, Washington, D. C.
2. Raetz, C. R. H. (1990) *Annu. Rev. Biochem.* **59,** 129–170
3. Raetz, C. R. H. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., ed) 2nd Ed., Vol. 1, pp. 1035–1063, American Society for Microbiology, Washington, D. C.
4. Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C. (eds) (1999) *Endotoxin in Health and Disease*, Marcel Dekker, Inc., New York
5. Raetz, C. R. H., and Whitfield, C. (2002) *Annu. Rev. Biochem.* **71,** 635–700
6. Galloway, S. M., and Raetz, C. R. H. (1990) *J. Biol. Chem.* **265,** 6394–6402
7. Onishi, H. R., Pelak, B. A., Gerckens, L. S., Silver, L. L., Kahan, F. M., Chen, M. H., Patchett, A. A., Galloway, S. M., Hyland, S. A., Anderson, M. S., and Raetz, C. R. H. (1996) *Science* **274,** 980–982
8. Jackman, J. E., Fierke, C. A., Tumey, L. N., Pirrung, M., Uchiyama, T., Tahir, S. H., Hindsgaul, O., and Raetz, C. R. H. (2000) *J. Biol. Chem.* **275,** 11002–11009
9. Clements, J. M., Coignard, F., Johnson, I., Chandler, S., Palan, S., Waller, A., Wijkmans, J., and Hunter, M. G. (2002) *Antimicrob. Agents Chemother.* **46,** 1793–1799
10. Zhou, Z., White, K. A., Polissi, A., Georgopoulos, C., and Raetz, C. R. H. (1998) *J. Biol. Chem.* **273,** 12466–12475
11. Doerrler, W. T., Reedy, M. C., and Raetz, C. R. H. (2001) *J. Biol. Chem.* **276,** 11461–11464
12. Karow, M., and Georgopoulos, C. (1993) *Mol. Microbiol.* **7,** 69–79
13. Clementz, T., Bednarski, J., and Raetz, C. R. H. (1995) *FASEB J.* **9,** 1311 (abstr.)
14. Clementz, T., Bednarski, J. J., and Raetz, C. R. H. (1996) *J. Biol. Chem.* **271,** 12095–12102
15. Polissi, A., and Georgopoulos, C. (1996) *Mol. Microbiol.* **20,** 1221–1233
16. van Veen, H. W., Callaghan, R., Soceneantu, L., Sardini, A., Konings, W. N., and Higgins, C. F. (1998) *Nature* **391,** 291–295
17. Putman, M., van Veen, H. W., and Konings, W. N. (2000) *Microbiol. Mol. Biol. Rev.* **64,** 672–693
18. Chang, G., and Roth, C. B. (2001) *Science* **293,** 1793–1800
19. Ambudkar, S. V., Lelong, I. H., Zhang, J., Cardarelli, C. O., Gottesman, M. M., and Pastan, I. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 8472–8476
20. Chang, X. B., Hou, Y. X., and Riordan, J. R. (1997) *J. Biol. Chem.* **272,** 30962–30968
21. Sun, H., Molday, R. S., and Nathans, J. (1999) *J. Biol. Chem.* **274,** 8269–8281
22. Ahn, J., Wong, J. T., and Molday, R. S. (2000) *J. Biol. Chem.* **275,** 20399–20405
23. Gorbulev, S., Abele, R., and Tampe, R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3732–3737
24. Miller, J. R. (1972) *Experiments in Molecular Genetics*, p. 433, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Paternostre, M. T., Roux, M., and Rigaud, J. L. (1988) *Biochemistry* **27,** 2668–2677
26. Gonzalez-Romo, P., Sanchez-Nieto, S., and Gavilanes-Ruiz, M. (1992) *Anal. Biochem.* **200,** 235–238
27. Brabetz, W., Muller-Loennies, S., Holst, O., and Brade, H. (1997) *Eur. J. Biochem.* **247,** 716–724
28. Dulbecco, R., and Vogt, M. (1954) *J. Exp. Med.* **99,** 167–182
29. Bligh, E. G., and Dyer, J. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
30. Raetz, C. R. H., and Kennedy, E. P. (1973) *J. Biol. Chem.* **248,** 1098–1105
31. Raetz, C. R. H., Purcell, S., Meyer, M. V., Qureshi, N., and Takayama, K. (1985) *J. Biol. Chem.* **260,** 16080–16088
32. Zhou, Z., Lin, S., Cotter, R. J., and Raetz, C. R. H. (1999) *J. Biol. Chem.* **274,** 18503–18514
33. Zhou, Z., Ribeiro, A. A., Lin, S., Cotter, R. J., Miller, S. I., and Raetz, C. R. H. (2001) *J. Biol. Chem.* **276,** 43111–43121
34. Nishijima, M., Bulawa, C. E., and Raetz, C. R. H. (1981) *J. Bacteriol.* **145,** 113–121
35. Radika, K., and Raetz, C. R. H. (1988) *J. Biol. Chem.* **263,** 14859–14867
36. Garrett, T. A., Kadrmas, J. L., and Raetz, C. R. H. (1997) *J. Biol. Chem.* **272,** 21855–21864
37. Carman, G. M., Deems, R. A., and Dennis, E. A. (1995) *J. Biol. Chem.* **270,** 18711–18714
38. Li, C., Ramjeesingh, M., Wang, W., Garami, E., Hewryk, M., Lee, D., Rommens, J. M., Galley, K., and Bear, C. E. (1996) *J. Biol. Chem.* **271,** 28463–28468
39. Mao, Q., Deeley, R. G., and Cole, S. P. (2000) *J. Biol. Chem.* **275,** 34166–34172
40. Osborn, M. J., Rick, P. D., and Rasmussen, N. S. (1980) *J. Biol. Chem.* **255,** 4246–4251
41. Nishijima, M., and Raetz, C. R. H. (1981) *J. Biol. Chem.* **256,** 10690–10696
42. Lawn, R. M., Wade, D. P., Garvin, M. R., Wang, X., Schwartz, K., Porter, J. G., Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) *J. Clin. Invest.* **104,** R25–R31
43. Oram, J. F., Lawn, R. M., Garvin, M. R., and Wade, D. P. (2000) *J. Biol. Chem.* **275,** 34508–34511
44. Wang, N., Silver, D. L., Costet, P., and Tall, A. R. (2000) *J. Biol. Chem.* **275,** 33053–33058
45. Bodzioch, M., Orso, E., Klucken, J., Langmann, T., Bottcher, A., Diederich, W., Drobnik, W., Barlage, S., Buchler, C., Porsch-Ozcurumez, M., Kaminski, W. E., Hahmann, H. W., Oette, K., Rothe, G., Aslanidis, C., Lackner, K. J., and Schmitz, G. (1999) *Nat. Genet.* **22,** 347–351
46. Brooks-Wilson, A., Marcil, M., Clee, S. M., Zhang, L. H., Roomp, K., van Dam, M., Yu, L., Brewer, C., Collins, J. A., Molhuizen, H. O., Loubser, O., Ouelette, B. F., Fichter, K., Ashbourne-Excoffon, K. J., Sensen, C. W., Scherer, S., Mott, S., Denis, M., Martindale, D., Frohlich, J., Morgan, K., Koop, B., Pimstone, S., Kastelein, J. J., and Hayden, M. R. (1999) *Nat. Genet.* **22,** 336–345
47. Rust, S., Rosier, M., Funke, H., Real, J., Amoura, Z., Piette, J. C., Deleuze, J. F., Brewer, H. B., Duverger, N., Denefle, P., and Assmann, G. (1999) *Nat. Genet.* **22,** 352–355
48. Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. (1999) *Annu. Rev. Pharmacol. Toxicol.* **39,** 361–398
49. Sauna, Z. E., Smith, M. M., Muller, M., and Ambudkar, S. V. (2001) *J. Biol. Chem.* **276,** 33301–33304
50. Oliver, D. B., Cabelli, R. J., Dolan, K. M., and Jarosik, G. P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 8227–8231
51. Borst, P., Zelcer, N., and van Helvoort, A. (2000) *Biochim. Biophys. Acta* **1486,** 128–144
52. Smit, J. J. M., Schinkel, A. H., Oude Elferink, R. P. J., Groen, A. K., Wagenaar,

[2] W. Konings, personal communication.

E., van Deemter, L., Mol, C. A. A. M., Ottenhoff, R., van der Lugt, N. M. T., van Roon, M. A., van der Valk, M. A., Offerhaus, G. J. A., Berns, A. J. M., and Borst, P. (1993) *Cell* **75,** 451–462
53. Francis, G. A., Knopp, R. H., and Oram, J. F. (1995) *J. Clin. Invest.* **96,** 78–87
54. Remaley, A. T., Schumacher, U. K., Stonik, J. A., Farsi, B. D., Nazih, H., and Brewer, H. B., Jr. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17,** 1813–1821
55. Sprong, H., van der Sluijs, P., and van Meer, G. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 504–513
56. Takayama, K., Qureshi, N., Mascagni, P., Nashed, M. A., Anderson, L., and Raetz, C. R. H. (1983) *J. Biol. Chem.* **258,** 7379–7385
57. Strain, S. M., Armitage, I. M., Anderson, L., Takayama, K., Qureshi, N., and Raetz, C. R. H. (1985) *J. Biol. Chem.* **260,** 16089–16098
58. Huijbregts, R. P., de Kroon, A. I., and de Kruijff, B. (1998) *J. Biol. Chem.* **273,** 18936–18942
59. Hrafnsdottir, S., and Menon, A. K. (2000) *J. Bacteriol.* **182,** 4198–4206
60. Kol, M. A., de Kroon, A. I., Rijkers, D. T., Killian, J. A., and de Kruijff, B. (2001) *Biochemistry* **40,** 10500–10506
61. Margolles, A., Putman, M., van Veen, H. W., and Konings, W. N. (1999) *Biochemistry* **38,** 16298–16306
62. van Veen, H. W., Venema, K., Bolhuis, H., Oussenko, I., Kok, J., Poolman, B., Driessen, A. J., and Konings, W. N. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10668–10672
63. Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S. D., and Sorokin, A. (2001) *Genome Res.* **11,** 731–753
64. Yakushi, T., Masuda, K., Narita, S., Matsuyama, S., and Tokuda, H. (2000) *Nat. Cell Biol.* **2,** 212–218